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The Stability System of the Yeast 2 Micron Plasmid: Analysis of Plasmid and Host Encoded Components

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The Stability System of the Yeast 2 Micron Plasmid: Analysis of Plasmid and Host Encoded Components

by

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Dedication

To my parents and my husband, Jijun

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The Stability System of the Yeast 2 Micron Plasmid: Analysis of Plasmid and Host Encoded Components

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Supervisor: Makkuni Jayaram

The work presented in this thesis aims to understand the molecular strategies used by an extrachromosomal selfish DNA element for its stable, high copy persistence. The model system studied here is the 2 micron plasmid found nearly ubiquitously in *Saccharomyces* yeast. By a combination of mutational and functional analysis of a plasmid coded protein Rep1p, an essential component of the stability system, we have provided support for the DNA-protein and protein-protein interactions predicted to be important in plasmid maintenance. Using cell biological and molecular genetic methods, we have unveiled an apparent coupling of the pathways for plasmid and chromosome segregation. Mutations that affect equal partitioning of the chromosomes also affect the plasmid, and the two tend to missegregate in tandem. We have identified host factors that interact with components of the plasmid stability system, and may thus play a potential role in

plasmid partitioning. In particular, we have found that the yeast cohesin complex, that bridges sister chromatids until they are ready to be unpaired and distributed to the daughter cells arising from a division event, may serve an analogous function in plasmid segregation. Our preliminary results suggest that the plasmid stability system follows the 'recruitment model', in which a functional complex is assembled by the sum of different sets of DNA-protein and protein-protein interactions. It is possible to reconstitute an active partitioning complex through an alternative set of interactions.

Table of Contents

List of Tables

List of Figures

CHAPTER 1

Introduction

1.1 Transmission of duplicated genetic information to progeny cells

Chromosome segregation, the process of equally distributing replicated genetic material into daughter cells, is a fundamental attribute of all living cells. In eukaryotes, active separation of paired sister chromatids to opposite cell poles during mitosis is achieved through the specific interactions between the mitotic spindle and centromeric regions of chromosomes (Barton and Goldstein, 1996; Moller-Jensen et al., 2000; Nicklas, 1997). Like their eukaryotic counterparts, bacteria also segregate their chromosomes efficiently and faithfully, only rarely giving rise to anucleate cells (Hiraga, 1992; Ireton et al., 1994; Mohl and Gober, 1997; Weitao et al., 2000). In addition to chromosomes, prokaryotes and eukaryotes can also harbor extrachromosomal elements. These include a variety of low or high copy plasmids found in bacteria (Actis et al., 1999; Moller-Jensen et al., 2000), the multi-copy DNA plasmid 2 micron circle (Volkert et al., 1989) and the double stranded killer RNA present in yeast. Similarly, some of the retrotransposons (Boeke and Devine, 1998; Sandmeyer, 1992; Wickner, 1992) as well as other repeated DNA elements associated with higher eukaryotic cells

(Buchowicz, 1997; Wilson and Williamson, 1997) go through a transient extrachromosomal phase. In addition, the genomes of certain mammalian viruses such as Epstein-Barr virus or papilloma virus exist predominantly in the plasmid state and are transmitted with high fidelity during cell division (Ilves et al., 1999; Voitenleitner and Botchan, 2002; Wu et al., 2000).

What are the mechanisms by which extrachromosomal DNA elements achieve efficient partitioning? If the copy number of an element is relatively high, random segregation will work just fine. For example, for a mean copy number of 20, according to Poisson distribution, the probability of a plasmid-free cell being formed during division is only 1.9×10^{-3}. The copy number within a given cell can then be adjusted to the steady state value through appropriate replication controls. For a low copy element, stable propagation is dependent on an active partitioning mechanism. If a unit copy element segregates by random distribution, roughly 30 percent of the cells would fail to inherit it per cell division.

The work presented here is focused on certain aspects of the partitioning functions encoded by the yeast plasmid. This plasmid presents a paradox in that it has a high steady state copy number, yet requires a partitioning system for stable propagation.

1.2 Plasmid and chromosome segregation in prokaryotes

Despite the identification and characterization of several genes involved in bacterial and plasmid DNA segregation, the molecular mechanisms by which these genes function have not been fully elucidated. In recent years, the use of cytological approaches to visualize the subcellular localization of proteins and DNA in live and fixed cells has contributed significantly towards our understanding of segregation mechanisms (Moller-Jensen et al., 2000). Plasmids as well as bacterial chromosomes display highly dynamic, yet ordered, localization patterns, signifying the presence of active mechanisms for segregation (Gordon et al., 1997; Jensen and Gerdes, 1999; Niki and Hiraga, 1997; Niki and Hiraga, 1998; Webb et al., 1998; Webb et al., 1997). Moreover, the intracellular localization of a number of proteins known to be involved in DNA segregation coincides with that of the DNA, suggesting that they are integral constituents of a partitioning machinery (Erdmann et al., 1999; Glaser et al., 1997; Jensen and Gerdes, 1999; Kim and Wang, 1998; Lin et al., 1997). The process of bacterial DNA segregation can be divided broadly into two stages: the decatenation and resolution of newly replicated plasmids or chromosomes to produce separable units, and their equipartitioning to each side of the divisional plane. This introduction will focus on the partitioning event.

1.2.1 Strategies for plasmid propagation in bacteria

The stable maintenance of low-copy-number episomal elements such as phage P1, the F factor and R1 plasmids are dependent on genetic loci present in these elements that either act in *cis* or encode *trans*-acting proteins (Gerdes et al., 1997; Hiraga, 1992; Jensen and Gerdes, 1995).

One class of such gene systems, called the proteic killer system, mediates plasmid maintenance by selectively killing plasmid-free cells (Jensen and Gerdes, 1995). These systems code for a stable toxin and an unstable antidote. The antidote prevents the lethal action of its cognate toxin by forming a tight complex with it (Ruiz-Echevarria et al., 1995; Tam and Kline, 1989). The differential decay rates of the toxin and the antidote appear to be the molecular basis for toxin activation in plasmid-free cells and their consequent lethality (Fig. 1.1). In two well-studied cases, *ccd* of F and *parD/pem* of R1/R100, the toxins interfere with the propagation of the chromosomes (Bernard and Couturier, 1992; Miki et al., 1992; Tsuchimoto and Ohtsubo, 1989). The proteic killer gene systems are summarized in Table 1.1.

Aside from the killer system, plasmids encode true partitioning loci that function by mediating the active distribution of equal number of plasmid copies to daughter cells (Hiraga, 1992; Nordstrom and Austin, 1989). In general, partitioning loci consist of three essential components: two genes encoding *trans*-acting proteins and a third *cis*-acting site (roughly analogous to eukaryotic centromeres)

Table 1.1 The components of the proteic killer gene systems in bacteria

Killler system	Killer protein	Antidote protein	Cellular target	Protease required for degradation of the antidote
ccd of F	CcdB (101aa)	CcdA (72aa)	Gyrase	Lon
parD/pem of R1/R100	Kid/PemK (110aa)	Kis/PemI (84aa)	DnaB	Lon
phd/doc of P1	Doc (126aa)	Phd (73aa)	Unknown	ClpXP

(Adapted from Jensen and Gerdes, 1995.)

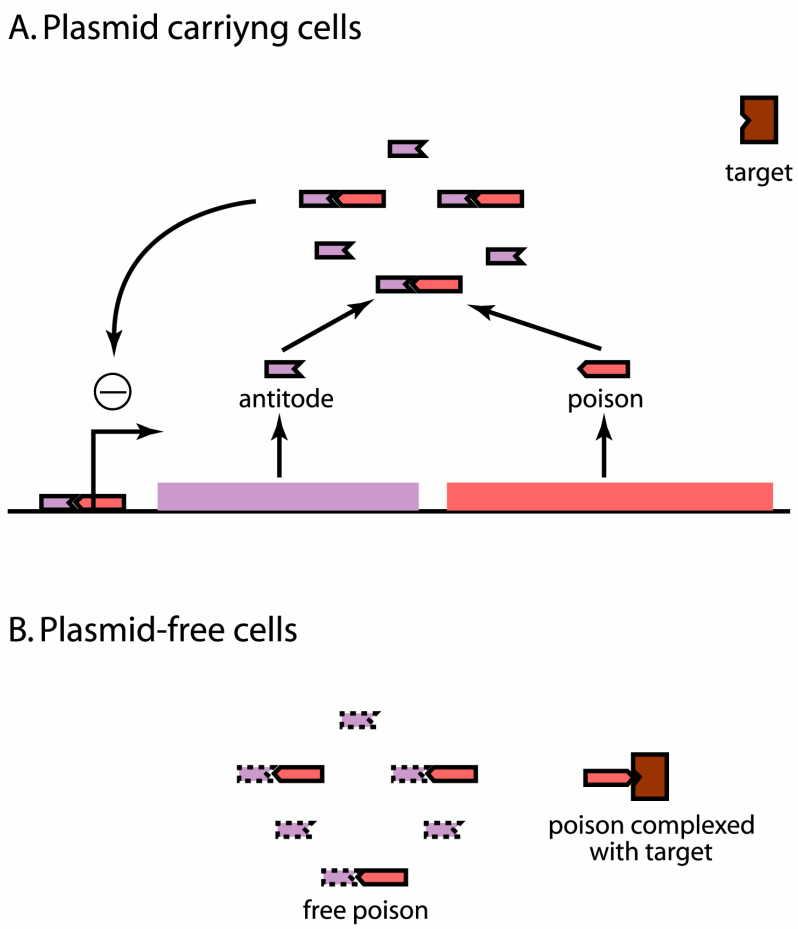


Figure 1.1 Schematic diagram showing the general structure of the proteic killer gene systems**.** A. They are organized as operons in which the antidotes are encoded by the upstream genes and the toxins by the downstream genes. The antidotes form complexes with the toxins, thereby neutralizing the latter in plasmid-carrying cells. In most cases, the operons are autoregulated by the toxin-antidote complexes, which bind to operators present in the promoter regions. B. The antidotes are degraded in plasmid-free cells, leading to activation of the toxins. (Adapted from Jensen and Gerdes, 1995)

to which the partitioning proteins bind. A brief summary of the well-studied plasmid partitioning systems is given in Table 1.2. In P1 and F, the genetic organization of the two partitioning loci is similar. The centromere-like sites, *parS/sopC*, are positioned immediately downstream of the co-transcribed partitioning genes, *parA/sopA* and *parB/sopB*. In R1, however, the centromere-like region, *parC*, is located upstream of the genes encoding the partitioning proteins ParM and ParR (Dam and Gerdes, 1994; Gerdes and Molin, 1986). For each, all the three elements are required for efficient plasmid segregation. ParB (P1), SopB (F) and ParR (R1) bind their centromere-like target site to form high-order nucleoprotein complexes (Davis and Austin, 1988; Jensen et al., 1998; Mori et al., 1989). ParA, SopA and ParM, which are ATPases (Bork et al., 1992; Koonin, 1993), make contact with the pre-formed partition complex and their ATPases activities are stimulated as a result of this contact (Bouet and Funnell, 1999; Jensen and Gerdes, 1997; Watanabe et al., 1989). Specific pairing of DNA molecules containing *parC* (in the R1 system) has been monitored by electron microscopy (Fig. 1.2; Jensen et al., 1998). However, ParM is different from ParA/SopA in two respects: it is a non-Walker-type ATPase while the other two belong to the family of Walker-type ATPases (Bork et al., 1992); it is not involved in regulation of its own promoter while ParA/SopA are (Davis et al., 1992; Hirano et al., 1998).

Table 1.2 Bacterial plasmid partitioning systems

Low Copy Plasmid	*Trans*-acting Proteins		*Cis*-acting element
	Non-ATPase	ATPase	
P1 plasmid	ParB	ParA	parS
F plasmid	SopB	SopA	sopC
R1 plasmid	ParR	ParM*	parC

* this is a non-Walker-type ATPase

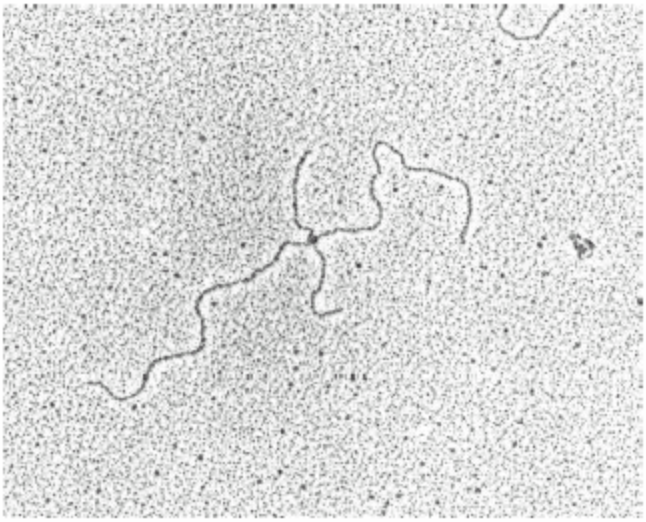


Figure 1.2 Electron micrograph of the nucleoprotein complex formed by ParM, ParR and *parC*. The complex holds together 2 plasmid molecules in preparation for their partitioning. A rough analogy may be drawn to sister chromatid cohesion in eukaryotes. (Adapted from Jensen et al., 1998)

With the use of fluorescent probes to visualize the subcellular localization of intracellular components, it appears that the partitioning of newly replicated DNA units occurs by rapid movement, rather than by a passive process coupled to the growth of the cell envelope (Gordon et al., 1997; Sharpe and Errington, 1998). As shown in Fig. 1.3, the F plasmid (also the P1 plasmid) is localized and replicated at the mid-cell position, and is subsequently moved rapidly to the quarter-cell positions in the elongated pre-division cell (Gordon et al., 1997). Following partitioning, the plasmids probably become tethered at these positions, which become the midpoints of the daughter cells (Moller-Jensen et al., 2000). An intact partitioning system is essential for the correct positioning of the plasmids as a function of the bacterial 'cell cycle' (Niki and Hiraga, 1997). The R1 plasmid localization (see Fig. 1.3) resembles that of P1 or F, albeit with one clear difference. In cells containing only one plasmid focus, it is located close to one pole or at mid-cell. However, in the majority of cells with two foci, the plasmids are positioned towards each of the two poles. One possible mechanism for R1 partitioning might be that plasmids are replicated at mid-cell and then rapidly shuttled to the cell poles, where they are tethered until completion of cell division. It has been shown by double labeling that SopB or ParB colocalizes with the corresponding plasmid. While the formation of ParB foci was dependent on the centromere-like element *parS*, its correct positioning was dependent on ParA (Erdmann et al., 1999; Kim and Wang, 1998). In contrast to the centromere-

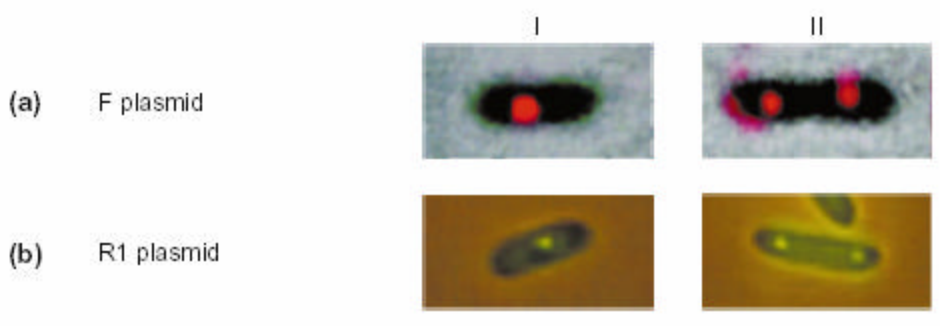


Figure 1.3 Intracellular localization of F and R1 plasmids. In (a), the plasmids were visualized using fluorescence *in situ* hybridization (FISH). And in (b), the plasmids were detected using a green fluorescent protein (GFP)-LacI fusion protein. Notice the translation of the plasmid foci from mid-cell (pre-replication; I) to quarter-cell positions (post-replication; II). (Adapted from Moller-Jensen et al., 2000)

binding proteins of P1 and F plasmids (ParB and SopB, respectively), ParR of the R1 plasmid does not exhibit specific cellular localization. However, ParM, the ATPase in the R1 partitioning system, forms distinct foci independently, and the plasmid co-localizes with ParM (Jensen and Gerdes, 1999).

1.2.2 Chromosome segregation in bacteria

1.2.2.1 *sop/par* homologues in chromosome segregation

Chromosomal homologues of the plasmid-encoded *sop/par* genes have been discovered in a wide range of bacterial species including *soj/spo0J* of *Bacillus subtilis* and *parAB* of *Caulobacter crescentus* (Gerdes et al., 2000; Ireton et al., 1994; Mohl and Gober, 1997). Like its plasmid-encoded orthologues SopB and ParB, Spo0J binds specifically to *cis*-acting DNA regions. Eight such regions, termed *parS*, have been identified in the origin-proximal 20% of the *B. subtilis* chromosome. When inserted into an unstable plasmid, a single *parS* region can stablilize the heterologous replicon, provided the partitioning proteins Soj and Spo0J are also present. Studies of Spo0J showed that this protein forms a pattern of discrete subcellular foci that colocalize with the chromosomal origin of replication revealed by co-immunodetection of Spo0J and BrdU-labelled origin (Lewis and Errington, 1997). The organization of Spo0J foci is dependent on Soj.

Upon initiation of replication, the Spo0J foci duplicate, and subsequently separate by rapid bi-directional movement towards the pole-proximal edges of the nucleoid. This movement closely parallels the localization dynamics of the *B. subtilis* chromosome: the origins move towards opposite poles after initiation of replication (Fig. 1.4), whereas the terminus remains at mid-cell. Thus, by direct interactions with the *parS* regions, Spo0J appears to form a compact nucleoprotein structure analogous to the plasmid partitioning complex. However, mutations that affect Spo0J function show only mild chromosome partitioning defects (Ireton et al., 1994). Furthermore, deletion of Soj (the SopA/ParA orthologue) has no effect on stable chromosome transmission, while at the same time markedly reducing the stability of *parS*-containing test plasmids. In addition, Soj represses sporulation-specific transcription in the absence of Spo0J. Overall these findings suggest that *soj/spo0J* constitute a molecular checkpoint linking DNA segregation to cellular development (Moller-Jensen et al., 2000).

In *C. crescentus*, the partitioning genes *parA* and *parB* are essential for cell viability. Purified ParB binds specifically to a region termed *parS*, located downstream of the *par* genes and within 80 kbp of the origin of replication (Fig. 1.5; Mohl and Gober, 1997). FISH experiments showed that in swarmer cells, the origin of replication is located at the flagellated pole and the terminus is positioned at the opposite pole. DNA replication takes place following the

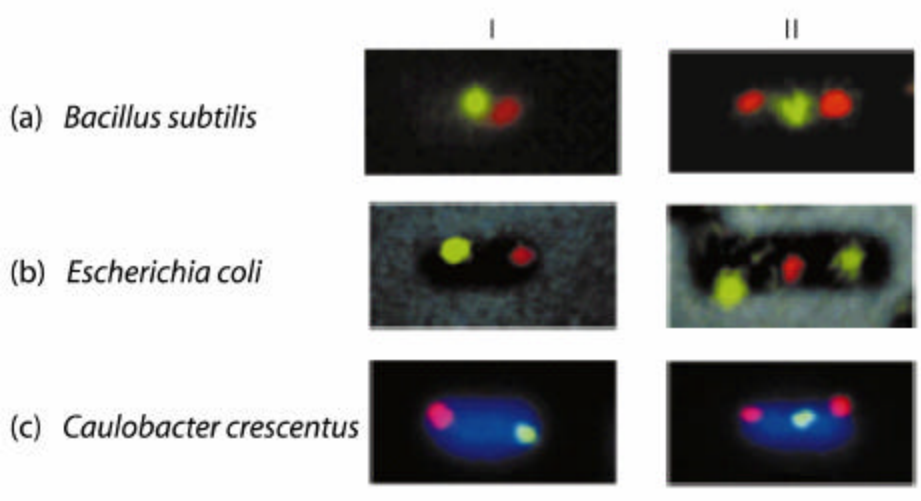


Figure 1.4 Intracellular localization of bacterial chromosomal DNA (the *oriC* and *terC* regions) before (I) and after (II) initiation of replication. (a) Localization of the *oriC* (red) and *terC* (green) regions of *Bacillus subtilis*. The position of the replication origin was visualized indirectly using fluorescently labeled antibodies against SpoOJ while the terminus was detected using GFP-LacI fluorescence. (b) Subcellular position of *Escherichia coli oriC* (green) and *terC* (red) detected using FISH. (c) Localization of origin (pink) and terminus (green) of *Caulobacter crescentus* using FISH. Nucleoids were visualized by DAPI staining (dark blue). (Adapted from Moller-Jensen et al., 2000)

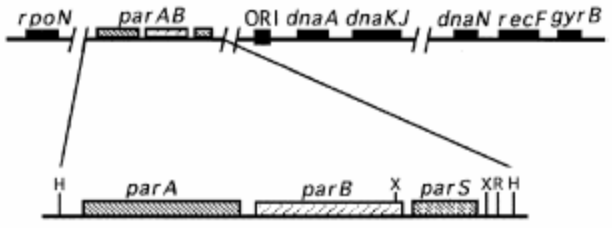


Figure 1.5 Genetic organization of the *C. crescentus parAB* Operon. The chromosomal location of *parAB* was mapped by hybridization of *parAB* coding DNA to restriction enzyme digested chromosomal DNA separated by pulse field gel electrophoresis. The *C. crescentus parAB* lies adjacent to the origin of replication (ORI). Immediately downstream of the *parAB* coding region is a noncoding DNA sequence, *parS*, which functions as a ParB binding site. H, HindIII; R, EcoRI; X, XhoI. (Adapted from Mohl and Gober, 1997)

differentiation of swarmer cells into stalked cells. Immediately after initiation of replication, one origin copy moves rapidly to the opposite pole followed by repositioning of the terminus to mid-cell (Fig. 1.4; Jensen and Gerdes, 1999). Both ParA and ParB localize towards the poles of the pre-divisional cells, and overexpression of either of the two partitioning proteins interferes with cell division and proper chromosome segregation. Thus, the *parAB* genes could serve both as part of a mitotic-like apparatus and as a cell-cycle check point coupling chromosome partitioning to cell division (Moller-Jensen et al., 2000).

1.2.2.2 SMC proteins in chromosome segregation

In slowly growing *Escherichia coli* cells, a single origin (*oriC*) focus forms at mid-cell (Niki et al., 2000). Following splitting of the single *oriC* focus into two, the foci move rapidly to opposite nucleoid borders, whereupon the terminus (*terC*) region is relocated to the middle of the nucleoid (Fig. 1.4). Duplicated termini are then separated at the final stage of partitioning (Niki and Hiraga, 1998). These events suggest the presence of an active mitotic-like apparatus in chromosome partitioning. However, a *sop/par*-like partitioning locus has yet to be identified in the *E. coli* chromosome. Instead, a genetic screen for *E. coli* mutants defective in chromosome partitioning revealed mutations in the *mukB* gene (Niki et al., 2000). MukB is probably functionally analogous to the

prokaryotic and eukaryotic SMC (Structural Maintenance of Chromosomes) proteins, which are involved in chromosome condensation and partitioning (further details are given below under section 1.3.1). Deletion of *mukB* in *E. coli* results in mutant phenotypes similar to those of *smc* mutants of *B. subtilis* and *C. crescentus*, namely, temperature-sensitive growth, nucleoid decondensation and chromosomal partitioning defects.

1.2.2.3 Replication in bacterial chromosome partitioning

The process of replication itself could act as a trigger for chromosome partitioning. Localization study of DNA polymerase in *B. subtilis* suggested that the replication apparatus (replisome) is located at fixed positions (Lemon and Grossman, 1998). This would imply that replicating DNA is actively spooled through a stationary 'replication factory' and that bi-directional extrusion of newly replicated DNA by the replisome could provide the force to partition nascent chromosomes (Moller-Jensen et al., 2000).

1.2.3 DNA partitioning models in bacteria

Based on recent studies (Jensen and Gerdes, 1997; Jensen et al., 1998; Niki and Hiraga, 1997; Niki and Hiraga, 1999), a simple plasmid partitioning

model is presented in Fig. 1.6(a) (Moller-Jensen et al., 2000). Plasmid replication at mid-cell leads to formation of a partitioning complex, in which the 'centromere-like' regions are paired with the help of plasmid-encoded partitioning proteins. Following completion of replication, an unknown mechanism actively separates the paired plasmid copies.

Partitioning of large chromosomes appears to be more complex than that of plasmids. Chromosomal members of the *sop/par* family of partitioning proteins could specifically interact with sequences near the origin of replication (Lin and Grossman, 1998; Mohl and Gober, 1997), and this might be responsible for formation of paired *oriC* regions and their subsequent attachment to what might be considered mitotic-like apparatus. During ongoing replication, spooling of replicating DNA through a stationary replication machinery might serve to drive sister nucleoids apart. Continuous condensation of newly synthesized DNA subsequently accounts for the bidirectional movement of bulk DNA towards opposite poles. This simplified model is shown in Fig 1.6(b) (Moller-Jensen et al., 2000).

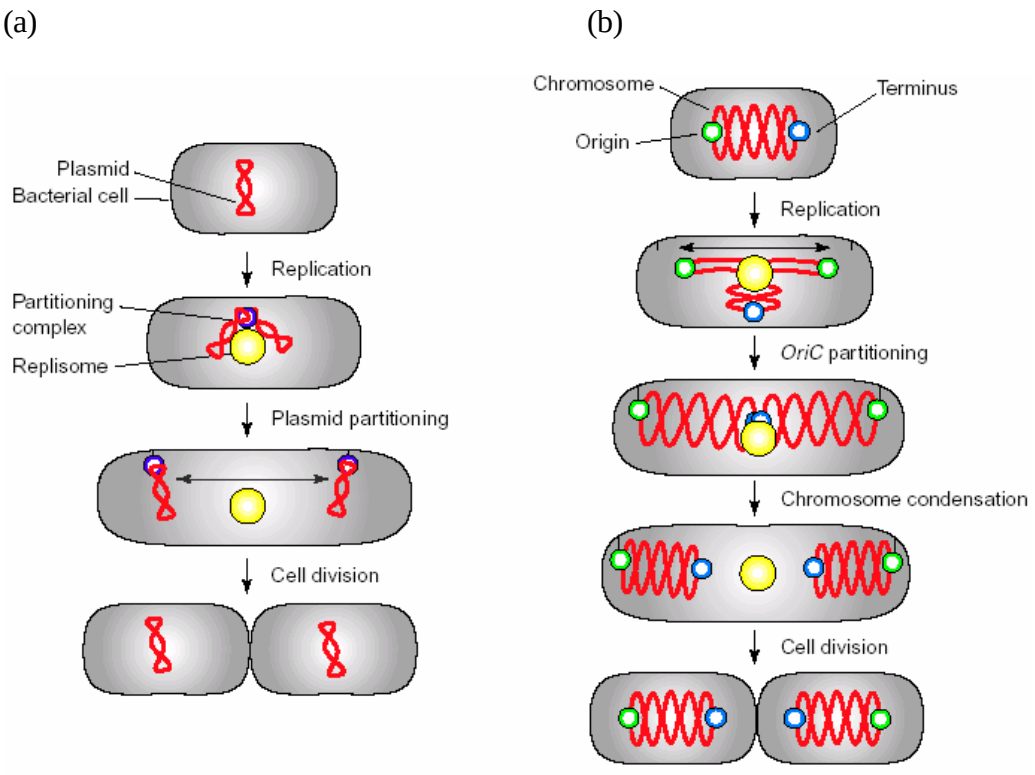


Figure 1.6 Plasmid and chromosome partitioning models in prokaryotes. (a) Plasmid partitioning. After replication at the centrally located replisome (yellow sphere) a partitioning complex where the partitioning proteins (purple sphere) bind to the plasmid centrosome-like region is formed. An unknown apparatus then rapidly moves the plasmid copies towards opposite cell poles. Subsequent tethering of the partitioned plasmids to specific positions might be mediated by the partitioning proteins. (b) Chromosome partitioning. Prior to replication, the chromosomal origin (green sphere) and terminus (blue sphere) are located at opposite edges of the compact nucleoid. During ongoing replication at mid-cell, the origins rapidly move towards opposite cell poles by unknown components. Bi-directional extrusion of newly replicated DNA from a stationary replisome and chromosome condensation account for bi-directional movement of bulk chromosomal DNA before cell division. (Adapted from Moller-Jensen et al., 2000)

1.3 DNA partitioning in eukaryotes

1.3.1 Brief review of chromosome segregation in yeast

Chromosomes go through several carefully timed and dramatic events during mitosis in eukaryotes. First, duplicated chromosomes are held together after DNA replication in S phase and throughout G2 phase (cohesion); Second, during the period spanning prophase to metaphase, an amorphous mass of interphase chromatin is condensed into compact units (condensation); Finally, at the onset of anaphase, the sister chromatids are split apart (separation) and rapidly dispatched to opposite cell poles. Recent biochemical and genetic studies have begun to shed light on the molecular mechanisms underlying these important processes during the mitotic cell cycle.

1.3.1.1 SMC proteins

Chromosome condensation and sister chromatid cohesion are regulated by protein complexes, termed condensin and cohesin respectively. One of the intriguing findings is that members of the SMC family (which are chromosomally acting ATPases) lie at the heart of these two protein complexes (Hirano, 2000).

The primary structure of SMC proteins, which is shared from bacteria to humans, consists of five distinct domains (Fig. 1.7). Two nucleotide-binding motifs, the Walker A and Walker B motifs, are located in the highly conserved N-terminal and C-terminal domains, respectively. The central domain is composed of a moderately conserved 'hinge' sequence that is flanked by two long coiled-coil motifs (Hirano, 2002). In eukaryotes, at least six members of the SMC protein family are found in individual organisms and each member forms an SMC heterodimer with a specific partner as Smc1p-Smc3p, Smc2p-Smc4p, and Smc5p-Smc6p. These heterodimers further associate with different sets of non-SMC subunits to assemble fully functional SMC holocomplexes (Table 1.3; Hirano, 2002). The most recent model on how two polypeptides are folded to make an SMC dimer came from the crystal structure of a bacterial SMC 'hinge' region along with EM studies and biochemical experiments on yeast Smc1 and Smc3 proteins (Fig. 1.8; Haering et al., 2002; Melby et al., 1998) Smc1p-Smc3p heterodimer is the core subunit of the cohesin complex, while Smc2p-Smc4p is that of condensin complex. Smc5p-Smc6p is believed to be involved in linking DNA repair and checkpoint responses, an aspect that will not be discussed here.

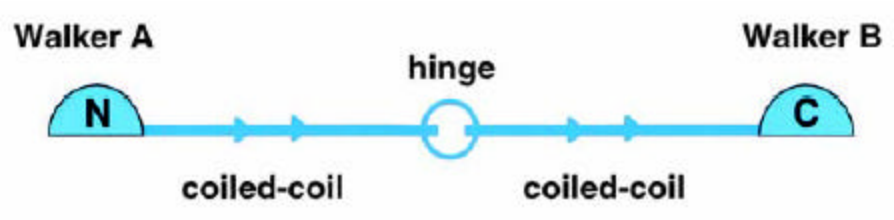


Figure 1.7 Primary structure of SMC proteins. The SMC monomer is a large polypeptide (between 1000 and 1400 amino acids). The N-terminal (~160 amino acids) and C-terminal (~150 amino acids) domains are highly conserved, and contain the nucleotide-binding Walker A and Walker B motifs, respectively. The central domain is composed of two long coiled-coil regions (between 300 and 350 amino acids) and a nonhelical hinge sequence (~200 amino acids). (Adapted from Hirano, 2002)

Table 1.3 Components of eukaryotic SMC protein complexes

Subunits	*S. cerevisiae*	*S. pombe*	*C. elegans*	*D. melanogaster*	*A. thaliana*	*X. laevis*	*H. sapiens*
Condensin							
SMC2	Smc2	Cut14	MIX-1	DmSMC2	BAB11491	XCAP-E	hCAP-E
SMC4	Smc4	Cut3	F35G12.8	DmSMC4/gluon	BAB10693	XCAP-C	hCAP-C
non-SMC	Ycs4	Cnd1	?	CG1911	CAB72176	XCAP-D2/Eg7	hCAP-D2/CNAP1
non-SMC	Ycs5/Ycg1	Cnd3	?	CG17054	BAB08309	XCAP-G	hCAP-G
non-SMC	Brn1	Cnd2	?	Barren	AAC25941	XCAP-H	hCAP-H
SMC4 variant	—	—	DPY-27	—	—	—	—
Cohesin							
SMC1	Smc1	Psm1	F28B34.7	DmSMC1	CAB77587	XSMC1	hSMC1α
SMC3	Smc3	Psm3	Y47D3A	DmSMC3/Cap	AAD26882	XSMC3	hSMC3
non-SMC	Scc1/Mcd1	Rad21	COH-1,2,3[a]	DmRAD21	>3 homologs	XRAD21	hRAD21
non-SMC	Scc3	Psc3[b]	F18E2.3	DmSA	CAB45374	XSA1 and XSA2	hSA1 and hSA2
SMC1β (meiotic)	—	—	—	—	—	?	hSMC1β
non-SMC (meiotic)	Rec8	Rec8	REC-8	?	SYN1/DIF1	?	Rec8
non-SMC (meiotic)	—	Rec11	?	CG13916?	?	?	STAG3/hSA3
SMC5-6 complex[c]							
SMC5	YOL034w	Spr18	C27A2.1	CG7783	CAC01791(MSS2)	AW638169 (est)	hSMC5
SMC6	Rhc18	Rad18	C23H4.6	CG5524	MIM	BG160113 (est)?	hSMC6

[a] COH-2 and COH-3 may have meiotic roles in the germ line.
[b] Psc3 is not tightly associated with the other cohesin subunits in *S. pombe*.
[c] This complex contains other subunits whose identities remain to be determined.
(Adapted from Hirano, 2002)

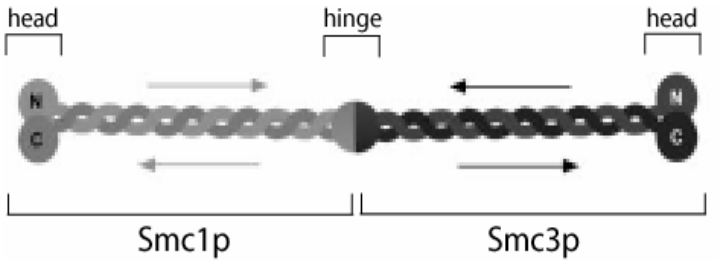


Figure 1.8 SMC dimmerization model. Individual SMCs form stable rod-shaped monomers containing a single coiled coil, with the hinge domain at one end and the globular head containing both N- and C- terminal domains at the other. These monomeric rods would be equivalent to one arm of the heterodimer. (Adapted from Haering et al., 2002)

1.3.1.2 Getting started---Establish sister chromatid cohesion during replication

In *Saccharomyces cerevisiae*, centromeric regions constitute one major set of sites at which cohesion between sister chromatids is established. The association between sisters extends several kilobases in each direction (Megee et al., 1999). Cohesin binding sites on chromosome arms occur at approximately 9 kb intervals, and tend to localize preferentially at AT-rich intergenic regions (Laloraya et al., 2000). These cohesin attachment regions, referred to as 'CARs', are typically 500-800 base pairs long.

Building cohesion between duplicated chromosomes appears to follow a multi-step pathway. In addition to the cohesin complex, consisting of Smc1p-Smc3p and the non-SMC components Scc1p-Scc3p, at least two other classes of proteins are also involved in this process, as shown by genetic studies in yeast: Eco1p/Ctf7p and Scc2p-Scc4p (Ciosk et al., 2000; Skibbens et al., 1999; Toth et al., 1999). In late G1, the cohesin core complex (Smc1p-Smc3p) is loaded onto 'pre-cohesion' sites on chromosome in an Scc2p/Scc4p dependent manner (Ciosk et al., 2000). Then the other cohesin subunits (Scc1p and Scc3p) bind CARs through Smc1p-Smc3p from early S until anaphase. The Eco1p/Ctf7p complex is necessary for establishing sister cohesion during S phase, gluing together the cohesin complexes bound on sister chromatids through Scc1p (also called Mcd1p; Skibbens et al., 1999; Toth et al., 1999). Although essential for the establishment

of cohesion, neither Eco1p/Ctf7p nor Scc2p-Scc4p is required in cohesion maintenance. The lethal defect in the *ctf7-203-ts* mutant can be suppressed at the non-permissive temperature by overexpression of PCNA (Skibbens et al., 1999), the processivity factor for a subset of the yeast DNA polymerases (Waga and Stillman, 1998). This observation suggests the possible involvement of replication fork components in the establishment of cohesion (discussed below).

The emerging model for cohesion is based on the observation that a novel DNA polymerase, Trf4/Pols (or Pol? as it was called earlier), is required for the establishment of the chromosome to chromosome bridge during the S phase (Fig. 1.9; Wang et al., 2000). This finding strongly supports the idea that replication fork components play an active role in this process with Trf4/Pols being a key link between the replication and cohesion machineries. According to this model, during replication, the core replicative polymerases would encounter a pre-cohesion site that has been occupied by components of the cohesin complex in late G1. This event is believed to initiate a switch from DNA pol α to DNA pol s within the replication complex. The fork components then would convert, by an unknown mechanism, a pre-cohesion site into a bona fide cohesion site at which the nascent duplexes are bridged to each other (Carson and Christman, 2001). In *S. cerevisiae*, Pol s is encoded by two redundant homologs, *TRF4* and *TRF5*

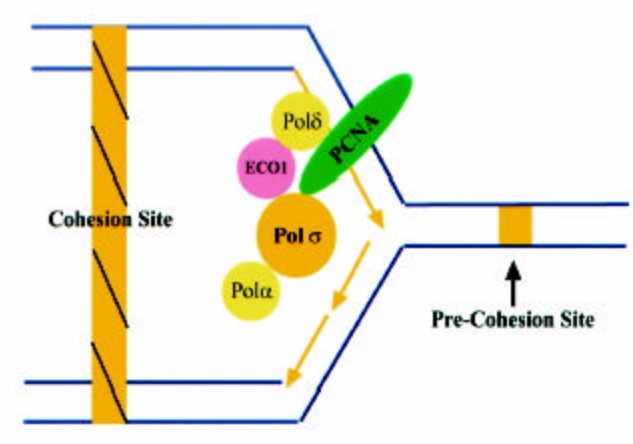


Figure 1.9 Model for establishment of cohesion between sister chromatids. Precohesin sites represent regions of the chromosome that, after passage of the replication fork, pair sisters via cohesin bridging. Components of the cohesin complex (Smc1 and Smc3 proteins, for example) are known to be associated with chromosomes even in the G1 phase of the cell cycle. The cartoon is not meant to imply the existence of direct protein-protein interactions, because such interactions have not been demonstrated to date. EcoIp (also called Ctf7p) is essential for establishing the cohesin bridge, but is not required for its maintenance. (Adapted from Carson and Christman, 2001)

(Castano et al., 1996a; Castano et al., 1996b; Sadoff et al., 1995). While a conditional *trf4 trf5* double mutant cannot complete DNA synthesis under non-permissive temperature, a single *trf4* mutant can go through an aberrant S phase with attendant cohesion defects.

1.3.1.3 Ready to go---Chromosome condensation and sister chromatid resolution

Mitotic chromosome condensation is a highly ordered and active process. A major breakthrough in the field over the past several years was the discovery of the condensin complex in frogs, flies and in yeast (Bhat et al., 1996; Hirano et al., 1997; Lavoie et al., 2000; Ouspenski et al., 2000; Saka et al., 1994; Steffensen et al., 2001; Sutani et al., 1999). The core subunits of the five-subunit yeast condensin complex are two SMC proteins, Smc2p and Smc4p. The other three non-SMC subunits are encoded by the *YCS4*, *YCS5* and *BRN1* genes (Table 1.3). All of the subunits are essential for mitotic chromosome condensation and cell viability, and are highly conserved from yeast to human (Uhlmann, 2001).

In vitro studies in *Xenopus* have contributed the most to shedding light on the plausible mechanisms for chromosome compaction by the condensin complex. The 13S condensin complex purified from *Xenopus* mitotic extracts binds directly to DNA in vitro and displays a DNA-stimulated ATPase activity. Moreover, positive supercoils can be introduced into a closed circular DNA by 13S

condensin as revealed by a topoisomerase I relaxation assay (Kimura and Hirano, 1997). The 13S condensin can also introduce knots into nicked circular DNA in the presence of a type II topoisomerase (Kimura et al., 1999). The sum of the results are consistent with condensin forming relatively large supercoiled loops by introducing global positive writhe in DNA in an ATP-dependent manner. Iteration of these positive supercoils can give rise to an ordered, solenoidal structure (Fig. 1.10; Kimura et al., 1999).

Since the DNA double helix is plectonemically coiled and chromosomes are not free floating linear DNA molecules but form topologically closed loops or domains, replication inevitably leads to interwined sister chromatids. Hence they have to be unlinked by decatenation mediated by topoisomerase II before they can be segregated (DiNardo et al., 1984; Holm et al., 1985). The positive writhing induced by condensin within a closed loop will be balanced by the generation of compensatory negative supercoils. In addition to stimulating the decatanation by topoisomerase II, these negative supercoils can further accentuate the degree of DNA compaction (Fig. 1.11; Hirano, 2000). Thus the condensation machinery is designed to ensure that the configuration of the segregating chromosomal entities is free from the hazards of entanglement and breakage that are inherent to long linear duplexes. Indeed, it was observed that barren, the *Drosophila* homolog of yeast condensin subunit Brn1p, coimmunoprecipitates with topoisomerase II and

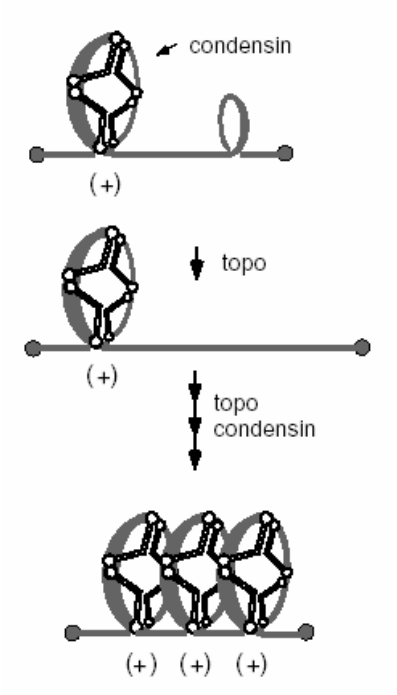


Figure 1.10 A model for chromosome condensation. In the global writhe model, condensin reconfigures DNA by introducing an ordered, global positive writhe (top left). In a closed DNA domain, compensatory negative supercoils will be formed (the loop on the right hand side). These may be relaxed by a topoisomerase, leaving a net gain in positive supercoiling. In this model, the compaction of DNA does not rely on the compensatory negative supercoil (middle), and condensin would play a more direct role in DNA organization (bottom). It is unknown how the global positive writhe might be introduced. One possible mechanism would be that multiple condensins make a positively supercoiled loop by introducing an array of nonplanar, right-handed bends in DNA, as shown here. Note that the model shows possible interactions between condensin and naked DNA and may not reflect the action of condensin on a chromatin fiber, in which case a lower stoichiometry of condensin-to-DNA appears to be sufficient to drive condensation. (Adapted from Hirano, 2000)

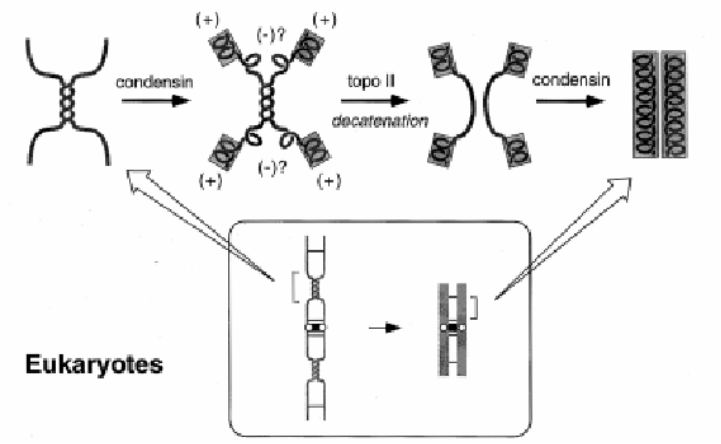


Figure 1.11 Chromosome condensation and resolution model. Condensin-mediated compaction of DNA during prometaphase may allow topoisomerase II (topoII) to resolve intertwined sister DNAs more efficiently. Local decatenation in turn allows more binding of condensins to the DNA and further promotes its compaction. After chromosome condensation is completed in metaphase, a certain level of DNA catenation remains between sister chromatids, although for simplicity it is not shown here. Full decatenation requires the continued action of topoisomerase II in anaphase. (Adapted from Hirano, 2000)

purified barren influences the activity of purified topoisomerase II *in vitro* (Bhat et al., 1996). However, this interaction has not been revealed in yeast and other systems. It is still an open question whether condensin and topoisomerase II function together via a direct physical interaction.

Commensurate with chromosome replication, formation of the cohesin bridge and DNA condensation, sister chromatids attach themselves to the spindle microtubules. This attachment is mediated through the kinetochore complex formed at the centromere region. The kinetochores of sister chromatids must face in opposite directions in order to facilitate their capture by microtubules from opposite cell poles (Shimoda and Solomon, 2002). Cells exercise a spindle checkpoint that forestalls the initiation of anaphase when proceeding through subsequent steps of the cell cycle would lead to chromosome missegregation. In brief, when there are kinetochores that are not under tension, which could result from the presence of a single mal-oriented or unconnected kinetochore, the onset of anaphase would be delayed (Li and Nicklas, 1995). One important regulator of this process is Ipl1p, which belongs to the family of aurora kinases (Bischoff and Plowman, 1999; Chan and Botstein, 1993; Cheeseman et al., 2002). By promoting microtubule turnover at the kinetochores, the Ipl1p activity gives sister kinetochores the chance to correct misorientations and attach themselves to microtubules from opposite poles (Tanaka et al., 2002).

1.3.1.4 Final separation

The separation of sister chromatids at the metaphase-to-anaphase transition is one of the most dramatic events of the eukaryotic cell cycle. Recent work in yeast has brought to light several important steps in this process (Ciosk et al., 1998; Nasmyth, 1999; Uhlmann et al., 1999). When cells are ready for chromosome segregation, the Anaphase Promoting Complex (APC), an ubiquitin ligase, is activated (Fang et al., 1999). As a result, the Pds1 protein (securin) is ubiquitinated and marked for degradation by the proteosome pathway (Cohen-Fix et al., 1996). In turn, the Esp1 endopeptidase (seperin) is released from securin (Pds1p), and dissociates the cohesin complex by cleaving the Scc1 subunit (Fig. 1.12; Ciosk et al., 1998; Nasmyth, 1999; Uhlmann et al., 1999). The dissolution of the cohesin bridge separates the sister chromatids and rapidly dispatches them towards opposite ends of the spindle apparatus.

Cytokinesis completes the division cycle to produce two daughter cells, each of which has a full complement of the genetic information of its parent.

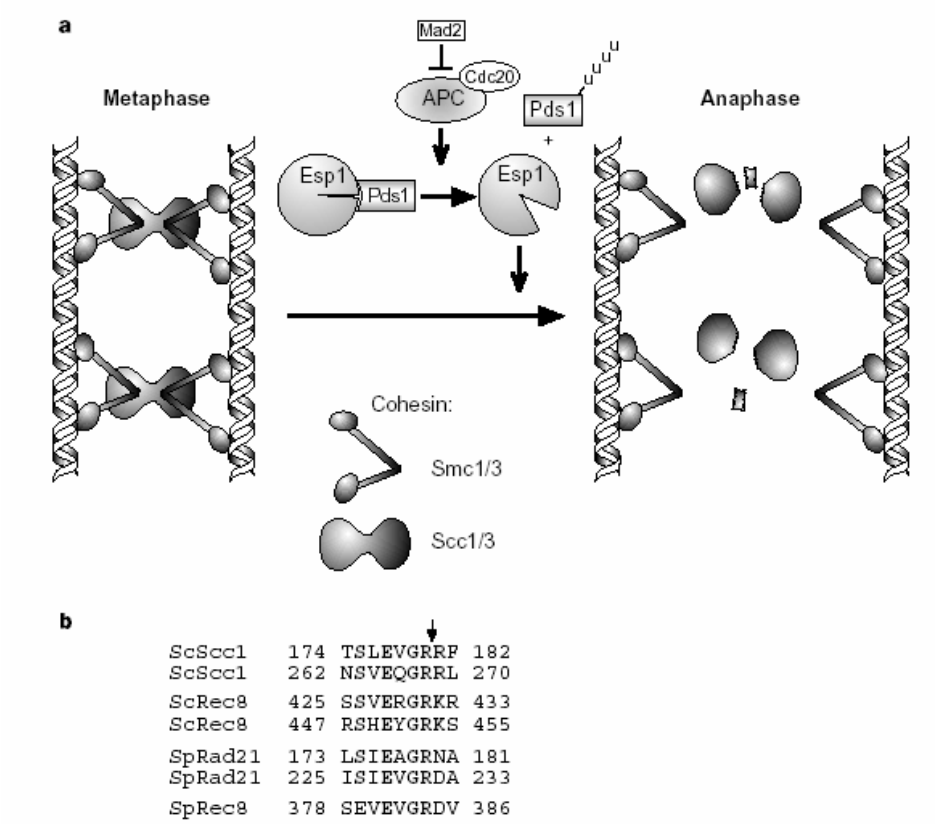


Figure 1.12 Model for separin action on cohesin and conservation of potential cohesin cleavage sites. a, Proteolytic cleavage of one of cohesin's subunits is necessary for sister separation, suggesting that cohesin complexes link sister chromatids. Further details are given in the text. Mad2 is included in the scheme as a known inhibitor of APC^{cdc20} (Amon, 1999). b, Alignment of known and putative cohesin cleavage sites. The arrow indicates the cleavage sites. (Adapted from Uhlmann et al., 1999)

1.3.2 2 micron plasmid segregation

The 2 micron circle plasmid, a 6318 bp double-stranded circular DNA molecule, is a multicopy extra-chromosomal element found in most strains of *Saccharomyces* yeast. The genetic make up of the plasmid is directed towards maintaining its steady state copy number (approximately 60 per cell) and mediating its stable propagation through the cell population (Broach and Volkert 1991). The general strategy used by the plasmid for stable, high copy propagation appears to follow the following three rules: 1. Effectively partition replicated plasmid molecules into daughter cells at cytokinesis; 2. Rapidly correct any decrease in copy number resulting from unequal segregation via an amplification mechanism (Futcher, 1986); 3. Institute provisions for positive and negative control on the amplification system to minimize fluctuations from the steady state plasmid density (Som et al., 1988).

The structural organization of 2 micron plasmid is shown in Fig. 1.13. In addition to the four open reading frames, namely *REP1*, *REP2*, *FLP* and *RAF*, the 2 micron circle harbors two regions of 599 bp inverted repeats, which separate the genome into two unique segments, 2774 bp and 2346 bp long (Broach et al., 1979; Hartley and Donelson, 1980). The plasmid contains a single autonomous replication sequence (*ARS/ORI*) that partially overlaps one of the repeated elements (Broach et al., 1983). Approximately 500 bp from *ORI* is a locus designated as *STB*, which includes of 5 to 6 inexact tandem repeats of a 62-bp

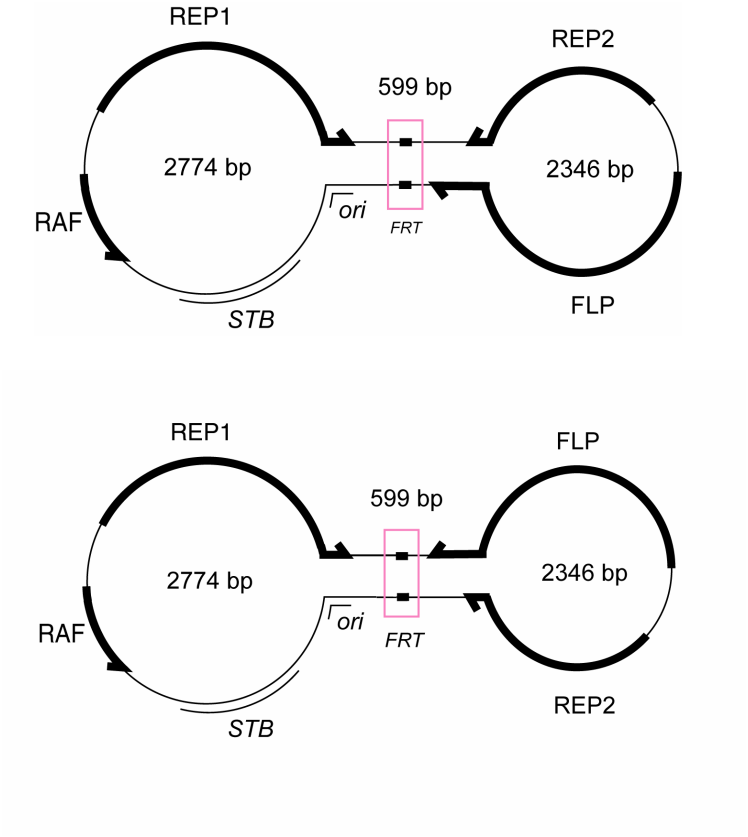


Figure 1.13 2 micron plasmid structure and gene arrangement. The locations of the four open reading frames are indicated by the heavy lines with arrows placed at the 3' ends of the genes. The *STB* element, *ori* and the *FRT* sites are also indicated. The recombination by Flp at *FRT* mediates relative inversion of the unique regions. The two forms of the 2 micron circle resulting from Flp protein mediated recombination are shown here.

sequence (Jayaram et al., 1983; Jayaram et al., 1985; Kikuchi, 1983). The Flp protein is a site-specific recombinase enzyme and acts on the *FRT* (Flp Recombination Target) sites to mediate relative inversion of the unique regions (Fig. 1.13; Broach et al., 1982). This reaction, coupled to plasmid replication is important in the copy number control of the plasmid (for further details, see below). The Rep1and Rep2 proteins, acting in conjunction with the *STB* locus, are responsible for maintaining the plasmid stably in the yeast cell population (Jayaram et al., 1983; Kikuchi, 1983). The Raf protein is poorly characterized, but is thought to play a regulatory role in fine-tuning the control of copy number (Murray et al., 1987; Som et al., 1988).

1.3.2.1 Rep1 protein, Rep2 protein and *STB* element in plasmid segregation

By using fluorescent probes to visualize the intracellular location and movement of 2 micron derived plasmids in live yeast cells, it has been shown recently that the plasmids exist as a cluster (most often in a tetrad configuration, although diads, triads and pentads are also seen) that persists throughout the cell cycle (Velmurugan et al., 2000). Following DNA replication, each of the duplicated cluster forms the unit of segregation, being rapidly pulled to opposite cell poles. The kinetics of plasmid and chromosome segregation closely parallels each other (Velmurugan et al., 2000). As already alluded to, the plasmid proteins

Rep1p, Rep2p and the *cis*-acting element *STB* are essential for normal partitioning. As is becoming evident from recent experiments, several chromosomally encoded factors also appear to contribute to the plasmid stability system. Further details regarding the role of the Rep proteins and host factors in plasmid maintenance constitute the central theme of the present work, and will be discussed in detail in the chapters to follow.

At a superficial level, the organization of 2 micron plasmid partitioning system has features similar to the partitioning systems of bacterial plasmids: two *trans*-acting proteins and a *cis*-acting element composed of tandem repeats of a consensus sequence (Jayaram et al., 1983; Kikuchi, 1983; Moller-Jensen et al., 2000). Analogous to the bacterial systems, interaction between the Rep proteins and *STB* has been established on the grounds of genetic, biochemical and cell biological evidence (Ahn et al., 1997; Scott-Drew and Murray, 1998; Sengupta et al., 2001; Velmurugan et al., 1998). And plasmid stability is severely compromised by mutations in either of the Rep proteins or by removal of the *STB* locus. However, despite the organizational similarity, so far there is no evidence for functional similarity between the yeast and bacterial systems. For example, no ATPase activity has been demonstrated for either Rep1p or Rep2p, and ATPase-specific sequence motifs are absent from their amino acid sequences.

The Rep1 protein is a 373-amino-acid polypeptide, based on DNA sequence (Broach et al., 1979), with a predicted size of 41.1 KD, although it runs

at approximately 48 KD in 10% denaturing SDS-polyacrylamide gels. In cell fractionation studies, Rep1p copurifies with an 'insoluble' subnuclear matrix/lamina/pore component. Secondary structure analysis predicts a bimorphic organization for a Rep1p protomer, with the amino-terminal half of the protein assuming a globular form and the carboxyl-terminal half assuming an extended a-helix. In addition, the a-helix portion of the molecule exhibits the heptad repeat pattern characteristic of proteins that form extended coiled-coil structures, such as vimentin, myosin heavy chain, and nuclear lamins A and C (Wu et al., 1987). Rep2 protein, 297 a.a. in length, is also a nuclear localized protein, and interacts with the Rep1 protein (more on these interactions later). The nuclear localizing sequences (NLS) within Rep1p and Rep2p have been mapped to their carboxyl-terminal extremities, and may be functionally substituted by exogenous NLS containing peptides (Velmurugan et al., 1998).

The *STB* locus is contained within the HpaI-AvaI segment of the large unique region of the plasmid, and can be divided roughly into two halves: *STB*-proximal and *STB*-distal (with respect to the origin). The directly repeated 5 to 6 copies of the 62 bp element, referred to earlier, are contained within *STB*-proximal (Murray and Cesareni, 1986). Two plasmid transcripts are terminated within *STB*-distal. The functionality of *STB* activity is affected by its local context. In particular, unrestricted transcription through the locus appears to diminish function, a property shared by other *cis*-acting elements, such as

centromeres and origins of replication (Hill and Bloom, 1987; Snyder et al., 1988).

Yeast plasmids lacking a partitioning or segregation system, *ARS* plasmids or *STB* plasmids in the absence of the Rep proteins, are transmitted inefficiently to daughter cells during mitosis and are frequently excluded from spore cells during meiosis (Murray and Szostak, 1983; Zakian and Scott, 1982). During mitotic division, there is a distinct maternal bias for plasmid retention, even for high copy number plasmids. Because of this asymmetric segregation, plasmid-free cells arise frequently and plasmids accumulate at high copy numbers in the remaining plasmid-bearing cells (mother cell lineage). Genetic analyses have suggested that Rep1p, Rep2p and *cis*-acting *STB*, acting in concert, contribute to the plasmid stability by overcoming the normal maternal bias (Jayaram et al., 1983; Kikuchi, 1983).

Self- and cross-interactions of Rep1p and Rep2p have been demonstrated by co-immunoprecipitation assays using extracts of *E. coli* cells that express them and by baiting assays using hybrid glutathione S-transferase (GST)-Rep proteins (Ahn et al., 1997). These findings were corroborated by carrying out *in vivo* dihybrid assays in yeast cells (Velmurugan et al., 1998). The interaction between the Rep proteins and the *STB* element, hypothesized a long time ago, received experimental support from the observation that urea-solubilized extracts from [cir^+] yeast cells (expressing Rep1p and Rep2p) could bind *STB* when assayed by

gel retardation. Extracts from [cir^0] cells did not contain the binding activity, but they became active when supplemented exogenously with Rep1p and Rep2p (Hadfield et al., 1995). The interaction has been confirmed and extended in the present study by using *in vivo* monohybrid assays in yeast (Velmurugan et al., 1998). Taken together, these observations suggest that a high-order complex formed by the Rep proteins and *STB* plays an active role in plasmid segregation.

1.3.2.2 Plasmid amplification system

A second strategy employed by the 2 micron plasmid for its stable maintenance is to amplify the copy number when it drops below the steady state value. Replication of the plasmid, carried out by the host replication machinery, is initiated at the origin, and proceeds bidirectionally (Brewer and Fangman, 1987; Huberman et al., 1987). The cell cycle control that precludes an origin from firing more than once per S phase applies to the plasmid as well (Zakian et al., 1979). Normally, each plasmid molecule replicates once, and only once, per cell cycle. However, when there is a decrease in copy number within a cell (due to a missegregation event, for example), the plasmid amplification system is set in motion to rectify the situation. The Flp site-specific recombinase and its target DNA sites (*FRT*) are central to the amplification reaction (Futcher, 1986; Mead et al., 1986).

The generally accepted model for amplification, proposed by Futcher, is illustrated in Fig. 1.14 (Futcher, 1986). According to the model, DNA replication will duplicate the *FRT* site proximal to the origin before duplication of the distal *FRT* site. A Flp-mediated recombination between one copy of the duplicated *FRT* and the non-duplicated *FRT* will change the direction of one of the two forks (which was bidirectional to begin with). The forks now assume a unidirectional configuration, and can traverse the circular template multiple times to spin out tandem copies of the plasmid. Thus, recombination coupled to replication can convert a single initiation event into a multiple copying mechanism. The cessation of amplification would require a second recombination event that can restore the normal direction of fork movement and replication termination. The time interval between the two successive recombination events would determine the degree of amplification. The iterated copies of the plasmid present in the concatamer can be resolved into single molecules by Flp-mediated recombination or by homologous recombination.

The amplification system is essentially a safety device that is normally kept under tight negative control to prevent run away increase in plasmid copy number, which would be harmful to the host. However, when needed, it must be

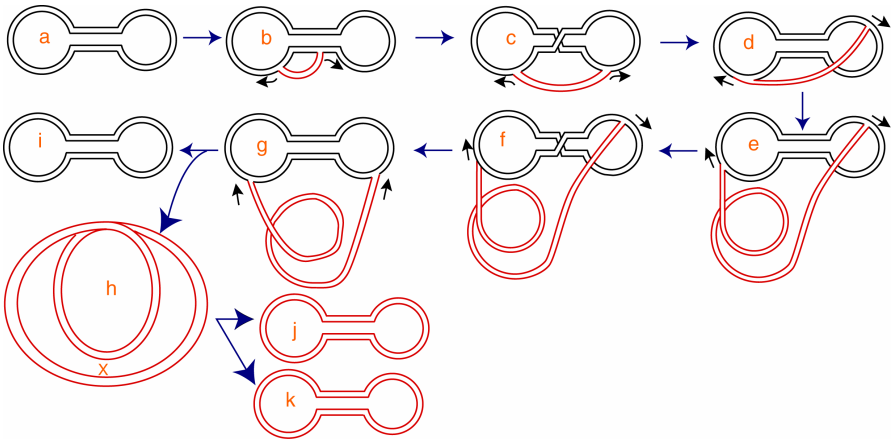


Figure 1.14 The Futcher model for 2 micron plasmid amplification. (a and b) DNA replication proceeds bidirectionally from the plasmid origin. Arrows indicate replication fork movement. (c and d) A recombination event mediated by Flp reorients the forks so that they no longer converge. (e) Continuing replication in this mode yields a multimeric replication intermediate. (f and g) A second Flp-mediated recombination event restores the converging orientation of the replication forks. Completion of replication yields a 2 micron circle monomer (i) and a multimer (h). Further Flp-mediated or general recombination resolves the multimer to monomers (j and k). (Adapted from Broach and Volkert, 1991)

turned on quickly in order to prevent downward drifts in copy number (see below, section 1.3.2.3; (Som et al., 1988).

1.3.2.3 Control of plasmid gene expression

The operation of a partitioning system for equal segregation of plasmids under normal growth conditions and the availability of an amplification system to compensate for rare missegregation events suggest that the plasmid is able to exquisitely sense fluctuations in copy number. Currently available data, primarily genetic and sparsely biochemical, are consistent with a mechanism in which counting plasmids and controlling copy number are accomplished by regulation at the transcriptional level (Som et al., 1988).

A general model for the regulatory circuit that controls 2 micron circle gene expression is presented in Fig 1.15. According to this scheme, Rep1p and Rep2p interact to form a repressor that negatively regulates transcription from all the 2 micron plasmid genes, except *REP2*. Given that *REP2* is constitutively expressed (and thus not limiting), the level of the Rep1p-Rep2p regulatory complex will be determined by *REP1* expression. Thus, the presumed bipartite repressor provides an indirect readout of the plasmid copy number. At or above a critical concentration of Rep1p-Rep2p, *FLP* expression would be turned off, silencing the amplification system. The *RAF* gene, which is thought to code for a

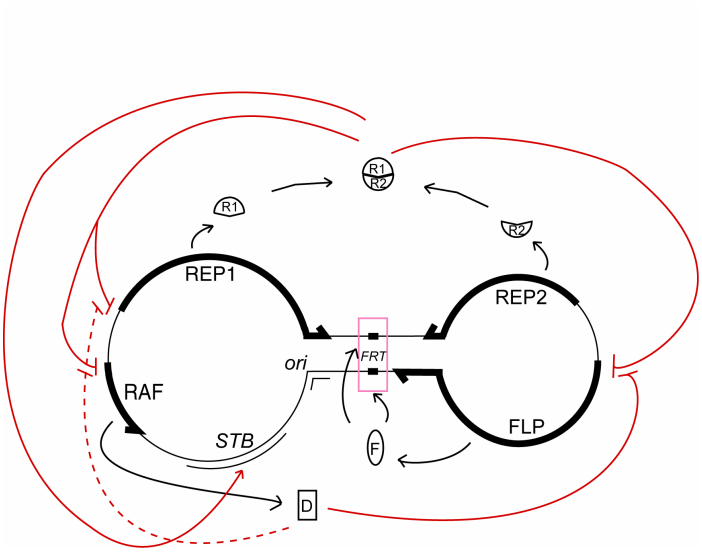


Figure 1.15 Proposed regulatory circuitry underlying plasmid partitioning and copy-number control. Interactions of 2 micron plasmid products with the plasmid genome are indicated. Flp (F) catalyzes recombination between specific sites *FRT*, a process required for plasmid copy number amplification. The products of *REP1* and *REP2* genes (R1 and R2) act in concert to promote equipartitioning at cell division via the *STB* locus. In addition, these products repress transcription of *FLP*, *REP1* and *RAF1*. Raf1p (D) antagonizes Rep1p/Rep2p-mediated repression of *FLP* gene expression, and perhaps of *REP1* and *RAF1* gene expression as well. (Adapted from Som et al., 1988)

positive regulator, is also turned off under this condition. The negative feed-back control of *REP1* transcription helps maintain the fluctuations in the Rep1p-Rep2p repressor concentrations within reasonable limits. The Raf1 protein, expressed at low plasmid copy number (or low repressor concentration) may facilitate a rapid amplification response by antagonizing Rep1p-Rep2p and thus inducing Flp. This antagonism would also upregulate Rep1p, reestablishing repressor levels and shutting off *FLP* and *RAF*. Thus, the fine-tuning of plasmid regulation ensures that Rep1p and Rep2p are made in amounts sufficient to sustain plasmid partitioning and to set the default state of amplification as 'off'. Furthermore, once triggered into action, amplification will be limited to short periods to prevent the copy number from overshooting the steady state value (Som et al., 1988).

1.3.2.4 Host factors involved in 2 micron plasmid segregation

Although the requirements for Rep1p, Rep2p and *STB* in plasmid partitioning have been known for some time (Jayaram et al., 1983; Kikuchi, 1983), the proposed models for their mode of action are tenuous at best. The suggested alternative mechanisms are (a) rendering the plasmid molecules freely diffusible and (b) actively distributing them to the daughter cells. Considering the molecular complexity of the eukaryotic mitotic machinery, it is rather hard to imagine that two proteins together with a relatively short DNA locus would be

sufficient to constitute an efficient partitioning system. It would seem almost certain that host-encoded components are also required for plasmid segregation. Consistent with this notion, recent studies have revealed the near equivalence between the chromosome and the 2 micron plasmid in the timing of their movement across the cell during the yeast cell cycle and the similarity in their segregation (Chapter 3; Velmurugan et al., 2000). A number of observations, which will be described in subsequent chapters, suggest that the Rep/*STB* system may be a clever molecular device for coupling plasmid partitioning to chromosome segregation.

The idea of coupling between chromosome and 2 micron plasmid segregation has been strengthened by the finding that, in several mutants affecting distinct steps of chromosome segregation, the plasmid almost always missegregates in tandem with the chromosome (Chapter 3 and Chapter 5; Mehta et al., 2002; Velmurugan et al., 2000). This chromosome-like behavior of the plasmid is absolutely dependent on the Rep/*STB* system. In the absence of the plasmid stability system, plasmid and chromosome segregations are uncoupled. Another intriguing fact concerns the recruitment of the yeast cohesin complex (an integral component of the chromosome segregation pathway) to the *STB* locus through the mediation of the Rep proteins. The periodicity of cohesin association and dissociation is nearly identical for the plasmid and the chromosomes in sequential cell cycles (Chapter 5; Mehta et al., 2002). The possibility that cohesin-

mediated pairing and unpairing likely provides a counting mechanism for evenly segregating plasmid molecules has to be entertained seriously.

1.4 Aims of the project and organization of chapters

The principal goal of my study is to further dissect the molecular mechanisms involved in the stable maintenance of the yeast 2 micron plasmid as a selfish extrachromosomal element in yeast. After describing the 'Materials and Methods' globally pertinent to this work in chapter 2, the major results are summarized in four subsequent chapters (Chapters 3-6).

The results summarized in Chapter 3 detail the organization and dynamics of the 2 micron plasmid in the yeast nucleus as a function of cell cycle progression. The plasmids exist as a tight cluster in association with the Rep1 and Rep2 proteins, and retain the clustered organization throughout the cell cycle. The integrity of the mitotic spindle appears to be important for the compactness of the plasmid cluster. Plasmids can be localized in chromosome spreads in a Rep1p and Rep2p dependent manner, and several chromosomal mutations that affect the fidelity of chromosome segregation also affect 2 micron circle segregation in a similar manner.

Chapter 4 is devoted to the analyses of functionally relevant DNA-protein and protein-protein interactions within the plasmid stability system. A library of

Rep1p variants was generated, and their ability to interact with Rep2p and *STB* as well as their competence in maintaining plasmid stability was examined. As was predicted from the working model, every Rep1p mutant that failed in either of the two interactions also failed to support normal plasmid partitioning. The assays also revealed a separate class of Rep1p mutants that was normal in these interactions, yet non-functional in plasmid maintenance. These are likely to encompass mutations that affect Rep1p-Rep1p self- interaction or interactions between Rep1p and host-encoded factors.

In Chapter 5, the results of a genome wide dihybrid assay in yeast to reveal host factors that interact with Rep1p are summarized. In addition, a number of aspects of the association between the Scc1p/Mcd1p subunit of the yeast cohesin complex and *STB* are dealt with as well. Two findings are particularly striking. First, the periodic cycling of cohesin at *STB* appears to be mediated by the cycling of the Rep proteins themselves at this locus. Second, the mitotic spindle likely plays a role in the association between cohesin and *STB*.

In chapter 6, we have addressed whether *STB* is a locus that is directly involved in the activation of the plasmid partitioning pathway or merely functions as a recruitment site for the Rep1 and Rep2 proteins. In these experiments, the *STB* locus was replaced by repeated copies of the *E. coli* LexA operator sequence. The ability of LexA-Rep1p and/or LexA-Rep2p hybrid proteins to support plasmid stability was then monitored.

As an epilogue, we briefly enumerate the significant findings made during this study, and outline future lines of investigation suggested by them.

CHAPTER 2

Materials and Methods

The materials and experimental procedures used in this study are summarized in this chapter. Specific variations of a particular procedure, where relevant, are described at the appropriate places in the text in individual chapters.

It is useful to note that yeast strains harboring endogenous 2 micron circles are indicated by [cir^+] and those cured of the circles by [cir^0]. Isogenic [cir^+] and [cir^0] strains provide convenient hosts for testing the maintenance behavior of an *STB*-containing plasmid in the presence of the Rep1 and Rep2 proteins or in their absence. For some of the experiments, the Rep proteins were expressed individually or in combination in a [cir^0] host strain, either from their native promoters or from strong constitutive (*ADH1*) or inducible (*GAL*) promoters.

2.1 General procedures

Yeast transformation was carried out by the lithium acetate protocol (Gietz et al., 1992). Standard genetic methods such as mating and tetrad dissection were performed as described by Rose et al., 1990. Bacterial transformations, plasmid

preparations, restriction enzyme digestions etc. were done as described by Sambrook et al., 1989.

2.2 Yeast strains and plasmids used in this study

The yeast strains and plasmids used in this study are listed in Table 2.1and Table 2.2. The alternative names by which some of the plasmids are referred to in the chapters comprising the 'Results' section are placed in parentheses. They better describe the plasmid configurations and/or convey specific features that are relevant to a particular experimental context. For example, pORI-OP0 and pORI-OP4 (described in Chapter 6) refer to plasmids containing the 2 micron circle origin, without LexA operators in one case (OP0) and four copies of the operator in the other (OP4).

2.3 Antibodies used in this study

Rep1 and Rep2 polyclonal antisera were generated in rabbits (provided by Dr. M. Dobson, University of Halifax). The antisera were affinity-purified, and tested for specificity prior to use. Anti-tubulin antibodies were obtained from Serotec (USA), Raleigh, NC. Polyclonal antiserum against the lac repressor protein was purchased from Stratagene, La Jolla, CA.

Table 2.1 Yeast strains used in this study

Strains	Genotype	Source	Reference
AFS479	MATa *ade2-1 ura3-1 trp1-1 leu2-3,112::lac(O)::LEU his3-?200::GFP-LacI::HIS3* [cir$^+$]	A. Murray	(Robinett et al., 1996)
CCY666-1A	MATa *ade2 ura3-52 leu2-3,112 his3-?200* [cir$^+$]	This study	
MJY30-10a	MATa *ade2 ura3-52 trp1-1 leu2-3,112 his3-?200::GFP-LacI::HIS3* [cir$^+$]	This study	
MJY31	MATa *ade2 ura3-52 trp1-1 leu2-3,112 his3-?200::GFP-LacI::HIS3* [cir^0]	This study	
CCY915-2B	MATa *lys2-801 ura3-52 trp1-1 leu2-3,112 his3-?200 ipl1-2* [cir$^+$]	C. Chan	(Kim et al., 1999)
MJY43	MATa *lys2-801 ura3-52 trp1-1 leu2-3,112 his3-?200 ipl1-2* [cir^0]	This study	
MJY130	MATa *ade-101 ura3-1 leu2-3,112 lys2-801 his3-?200 ctf13-30* [cir$^+$]		
MJY131	MATa *ade-101 ura3-1 leu2-3,112 trp1 his3-?200 ctf14-42* [cir$^+$]		
MJY132	MATa *ade-101 ura3-1 leu2-3,112 trp1 his3-?200 ctf7?-1::HIS3 ctf7-203::LEU2* [cir$^+$]		
MJY133	MATa *ade-101 ura3-1 leu2-3,112 trp1 ndc10-2* [cir$^+$]		
MJY134	MATa *ade-101 ura3-1 leu2-3,112 trp1 his3-?200 ndc80-1* [cir$^+$]		
FVY889-566	MATa *ade2 leu2-3,112 ura3-52 his5-2* [cir0]; Gal$^+$	This study	
PJ69-4A	MATa *ade2 ura3 trp1 leu2-3 his3 gal4? gal80? GAL1-HIS3 GAL2-ADE2 GAL7-LacZ* [cir$^+$]	P. James	(James et al., 1996)
EGY48	MATa *trp1 ura3-52 his3 leu2::3Lexop-LEU2* [cir$^+$]	R. Brent	(Finley and Brent, 1996)
YM4271	MATa *lys2-801 ade2-101 leu2-3,112 ura3-52 trp1 his3-?200 gal4? gal80?* [cir$^+$]	Clontech	
CRY1	MATa *ade2-1 ura3-1 leu2-3,112 trp1 his3-11* [cir$^+$]	I. Ouspenski	(Ouspenski et al., 2000)
CRY1-0	MATa *ade2-1 ura3-1 leu2-3,112 trp1 his3-11* [cir^0]	This study	
YIL178-20	MATa *ade2-1 ura3-1 leu2-3,112 trp1 his3-11 brn1-20* [cir^0]	I. Ouspenski	(Ouspenski et al., 2000)
YIL178-34	MATa *ade2-1 ura3-1 leu2-3,112 trp1 his3-11 brn1-34* [cir^0]	I. Ouspenski	(Ouspenski et al., 2000)
YIL178-60	MATa *ade2-1 ura3-1 leu2-3,112 trp1 his3-11 brn1-60* [cir$^+$]	I. Ouspenski	(Ouspenski et al., 2000)
BY4739	MATa *leu2 lys2 ura3* [cir$^+$]	Invitrogen	
FUN30del	MATa *leu2 lys2 ura3 fun30::KanMx* [cir^0]	Invitrogen	
MJY92	MATa *ade2 leu2 ura3 trp1 his3::GFP-LacI::HIS3* [cir^0]	This study	
MJY110	MATa *ade2-101 ura2-1 leu2-3,112 trp1::MCD1-3HA::URA3* [cir$^+$]	This study	
MJY111	MATa *ade2-101 ura2-1 leu2-3,112 trp1::MCD1-3HA::URA3* [cir^0]	This study	
MJY146	MATa *ade2-1 ura3-1::TUB1-YFP::URA3 leu2-3,112 trp1 his3-11 ::MCD1-3HA::KanMx* [cir$^+$]	This study	

Table 2.2 Plasmids used in this study

Plasmids	Vector type/Marker	Salient feature
pSV1	YEp/*URA3*	lac(O) repeats cloned in YEpLac195
pSV2	YCp/*LEU2*	lac(O) repeats cloned in YCpLac111
pSV3	YIp/*TRP1 URA3*	lac(O) repeats cloned in YRp17
pUC19-REP1	*E. coli* plasmid	REP1 cloned in pUC19
pTS408-REP1	YCp/*URA3*	Expressing GFP-Rep1p (Gal-inducible)
pTS408-rep1	YCp/*URA3*	Expressing GFP-Rep1p variants (Gal-inducible)
cp20 (pSTB)	YEp/*LEU2 ADE2*	Reporter for plasmid stability
cp21 (pSTB-REP1)	YEp/*LEU2 ADE2*	Expressing Rep1p (native promoter and terminator)
cp22 (pSTB-REP2)	YEp/*LEU2 ADE2*	Expressing Rep2p (native promoter and terminator)
pTS408-CterR1	YCp/*URA3*	Expressing GFP-Cter (last 25 aa's)Rep1p (Gal-inducible)
pTS408-CterR2	YCp/*URA3*	Expressing GFP-Cter (last 25 aa's)Rep2p (Gal-inducible)
pGBDUc1-SREP2	YEp/*URA3*	Expressing Gal4BD-SRep2p (*ADH1* promoter)
pGBDUc1-REP1	YEp/*URA3*	Expressing Gal4BD-Rep1p (*ADH1* promoter)
pGADc1-REP1	YEp/*LEU2*	Expressing Gal4AD-Rep1p (*ADH1* promoter)
pGADc1-REP2	YEp/*LEU2*	Expressing Gal4AD-Rep2p (*ADH1* promoter)
pGAD424-rep1	YEp/*LEU2*	Expressing Gal4AD-Rep1p variants (*ADH1* promoter)
pEG202-REP1	YEp/*HIS3*	Expressing LexA-Rep1p (*ADH1* promoter)
pEG202-REP2	YEp/*HIS3*	Expressing LexA-Rep2p (*ADH1* promoter)
pJG4-5-REP1	YEp/*TRP1*	Expressing AD*-Rep1p (Gal-inducible)
pJG4-5-rep1	YEp/*TRP1*	Expressing AD*-Rep1p variants (Gal-inducible)
2μ-ADE2	YEp/*ADE2*	*ADE2* inserted into native 2 micron circle
2μ-ADE2(flp)	YEp/*ADE2*	Similar to 2μ-ADE2, except that Flp is disrupted by *ADE2* insertion
pBM272-REP1Y43A	YCp/*URA3*	Expressing Rep1Y43A (Gal-inducible)
pBM272-REP1K297Q	YCp/*URA3*	Expressing Rep1K297Q (Gal-inducible)
pEG202-BRN1	YEp/*HIS3*	Expressing LexA-Brn1p (*ADH1* promoter)
pJG4-5-BRN1	YEp/*TRP1*	Expressing AD*-Brn1p (Gal-inducible)
pGAD424-BRN1	YEp/*LEU2*	Expressing Gal4AD-Brn1p (*ADH1* promoter)
pJG4-5-FUN30	YEp/*TRP1*	Expressing AD*-Fun30p (Gal-inducible)
pGAD424-MCD1	YEp/*LEU2*	Expressing Gal4AD-Mcd1p (*ADH1* promoter)
pESC-TRP-MCD1	YEp/*TRP1*	Expressing Myc-Mcd1p (Gal-inducible)
YEpLac195-REP1-REP2	YEp/*URA3*	Expressing Rep1p and Rep2p (native promoters and terminators)
YEpLac181-REP1-REP2	YEp/*LEU2*	Expressing Rep1p and Rep2p (native promoters and terminators)
pBTM116-REP2	YEp/*TRP1*	Expressing LexA-Rep2p
pAA0 (pORI-OP0)	YIp/*URA3*	---
pAA4 (pORI-OP4)	YIp/*URA3*	LexA(O) repeats cloned in pAA0
pAA4c (pORI-OP4-1)	YIp/*URA3*	LexA(O) repeats cloned in pAA0, close to *ORI*
pAA4d (pORI-OP4-2)	YIp/*URA3*	same as pAA4c except repeats in opposite orientation
pBM272-REP1	YCp/*URA3*	Expressing Rep1p (Gal-inducible)
pBM272-REP2	YCp/*URA3*	Expressing Rep2p (Gal-inducible)
pBM272-REP1-REP2	YCp/*URA3*	Expressing Rep1p and Rep2p (Gal-inducible)
pBM272TRP-REP1	YCp/*URA3*	Expressing Rep1p (Gal-inducible)
pBM272TRP-LexAREP2	YCp/*URA3*	Expressing Rep2p (Gal-inducible)
pBM272TRP-REP1-LexAREP2	YCp/*URA3*	Expressing Rep1p and Rep2p (Gal-inducible)

*AD, acid-rich transcriptional activation domain.

2.4 Synchronization of yeast cells in G1 phase by a-factor (Chapter 3)

Cells were grown overnight in selective medium, washed and resuspended either in the same medium or YEPD at an OD_{600} of approximately 0.1. The culture was incubated at 30°C for 90 min., and α-factor was added to a final concentration of 7μg/ml. Incubation at 30°C was continued for 3 hr., and the percentage of cells arrested in G1 was monitored by microscopy (Breeden, 1997).

2.5 Nocodazole treatment

An exponentially growing yeast culture was treated with either 1% DMSO (control) or 20 μg/ml nocodazole (Sigma) in 1% DMSO. The cells were incubated in the presence of the drug at 30°C for 2 hr. Examination of the cells under the microscope revealed approximately 85% of them to be arrested with large buds.

2.6 *In vivo* visualization of plasmids or chromosome (Chapter 3)

The yeast strain(s) containing the expression cassette for GFP-lac repressor was transformed with the appropriate reporter plasmid(s) containing the lac operator repeats. The expression of the hybrid repressor was induced by the

addition of 10 mM 3-AT (3-aminotriazole) for 30 min. The lac operator DNA bound by GFP-repressor was visualized by fluorescence microscopy following excitation at the appropriate wavelength. In order to obtain optimal fluorescence, the pH of the media was maintained at 6.5 by the addition of trisodium citrate (6.5 g per liter). Cells were observed under an Olympus BX-60 microscope with recommended filters for GFP excitation and emission. Images were captured using a Spot Digital Camera from Diagnostic Instruments, and were processed using Image-Pro Plus software from Media Cybernetics. Confocal images were taken using the Leica confocal system, TCS4D (Core Facility, Institute for Cell and Molecular Biology, UT Austin).

2.7 Immunofluorescence assay (Chapter 3)

Yeast cells grown to mid log phase (10^6 cells per ml) were fixed in 5% formaldehyde solution for 60 min. at room temperature. The fixed cells were washed once with PBS (phosphate buffered saline), once with 1.2 M sorbitol/1mM EDTA, and resuspended in the same medium to a final density of 10^8 cells per ml. Spheroplasts were obtained by incubating with 1 mg/ml of zymolyase 100T (US Biologicals, Swampscott, MA) in the presence of 10% β-mercaptoethanol for 60 min at 30°C. The spheroplasts were washed with PBS, transferred to poly-L-lysine coated slides, and flattened on them using methanol

(5 min.) and acetone (30 sec.). Immunofluorescence staining was done according to Adams and Pringle, 1984 with some modifications. Blocking was done using 1 mg/ml BSA for 15 min. All the primary and secondary antibodies were diluted in the antibody dilution buffer (1 mg/ml BSA and 0.02% sodium azide in PBS). Incubations with primary and secondary antibodies were done at room temperature for 60 min. and 30 min., respectively. Observations were made after mounting the samples using mounting solution supplied by KPL Laboratories (Gaithersburg, MD). Microscopy was carried out using an Olympus BX-60 microscope or the Leica Confocal System, TCS4D. Images were taken at 100X magnification and processed in Image-Pro Plus (media Cybernetics) or PhotoShop 5.0 (Adobe Systems Incorporated) software.

2.8 Chromosome spreads (Chapter 3)

Chromosome spreads from mitotic cells were prepared by following the procedure of Nairz and Klein, 1997 with minor modifications. 20 µl of the yeast spheroplasts were mixed gently with 40 µl of 4% paraformaldehyde/3.4% sucrose and 80 µl of 1% lipsol, and spread on glass slides. After overnight incubation at room temperature, the slides were washed twice with 0.4% photoflo-200 (Kodak, NY) and once with 1 x PBS. Then the chromosome spreads were first blocked with 1 mg/ml BSA for 15 min. at room temperature. Primary antibody was added

and incubated in a humid chamber at room temperature for 3 hrs. The slides were washed with 1xPBS, and incubated with the secondary antibody conjugated to a fluorescent dye for 1hr at 26^0C. 1 µg/ml DAPI in 1xPBS was used as the DNA stain. Slides were mounted with mounting media and cover glass, and examined by fluorescence microscopy.

2.9 *Z*-series sectioning of yeast nucleus (Chapter 3)

The compactness (or the residence zone) of plasmid clusters within yeast nuclei was determined by *z*-series sectioning of the yeast nucleus using confocal microscopy. For each sample, 40 sections at 0.25 µm thickness were examined, spanning 5 µm of total thickness. The start point for scanning was set manually approximately 2 to 3 frames beyond the boundary of fluorescence from the GFP-lac repressor tagged plasmid. Thereafter, the same number of sections (or the same total distance) was scanned for each sample. In every case, the set range completely covered the limits of the plasmid fluorescence zone. An identical procedure was used to obtain the boundary range of the DAPI staining region in each of the cells examined. The ratio of the green fluorescence range to the blue fluorescence range was calculated for each cell. Values from at least 20 individual cells were pooled to express the mean width (+/- S.D.) of the plasmid residence zone.

2.10 Assay for plasmid segregation in host strains harboring the *ipl1-2* mutation (Chapter 3)

The yeast strains were grown in appropriate selective media at 26°C, and were arrested in G1 by α-factor treatment. After washing away the pheromone, the cells were allowed to recover from growth arrest at 26°C for 90 min. They were then shifted to 37°C and allowed to grow for 4 hr. DAPI was added to the growth medium (final concentration of 2μg/ml), and cells were harvested 30 min later. They were washed with sterile water, fixed in 3.5% formaldehyde (0-4°C), and observed under the microscope. Roughly 75-80 percent of the cells in the population contained large buds. Plasmid and chromosome segregation data shown in Fig. 3.5 pertain only to the large-budded cells.

2.11 Site-specific mutagenesis of Rep1 protein by PCR (Chapter 4)

Mutagenesis was carried out as illustrated in Fig. 2.1 on the *REP1* gene cloned in pUC19. The plasmid was prepared from the *E. coli* strain DH5a, such that the DpnI sites were fully methylated and would be susceptible to digestion by DpnI. For a specific amino acid position, a pair of mutagenic oligos was used in a PCR-directed strategy to obtain a library of mutants. Each of the two oligos had the wild type *REP1* sequence, except at the targeted triplet position, which was randomized in each oligo by including an equimolar mixture of all four

phosphoramidites during synthesis (indicated as NNN/N'N'N" in Fig. 2.1). The oligos were fully complementary to each other in the regions flanking the randomized triplet. The full length pUC19-REP1 was PCR amplifed using oligos one and two as primers. Note that the vast majority of the amplification product would be unmethylated in both strands and therefore resistant to the action of DpnI. A small amount of the products would be hemi-methylated, corresponding to semiconservative replication of the parental template strands. Any unreplicated parental DNA (methylated on both strands and representing only a tiny mole fraction of the DNA population) will be cut by DpnI. After treating with DpnI to selectively enrich the desired PCR product (circularized via the cohesive oligo ends), DNA was transformed into *E. coli* DH5a. The endogenous ligase was expected to covalently close the cohesive ends and generate circular plasmids harboring *rep1* mutants. Plasmids were isolated from the *E. coli* transformants and subjected to DNA sequencing to identify the mutations.

2.12 Yeast dihybrid assay (Chapters 4, 5)

The dihybrid assays were carried out according to procedures described by Finley and Brent (1996) and/or by P. James (James et al., 1996). The two systems are based on the same principle that the interaction between two protein partners

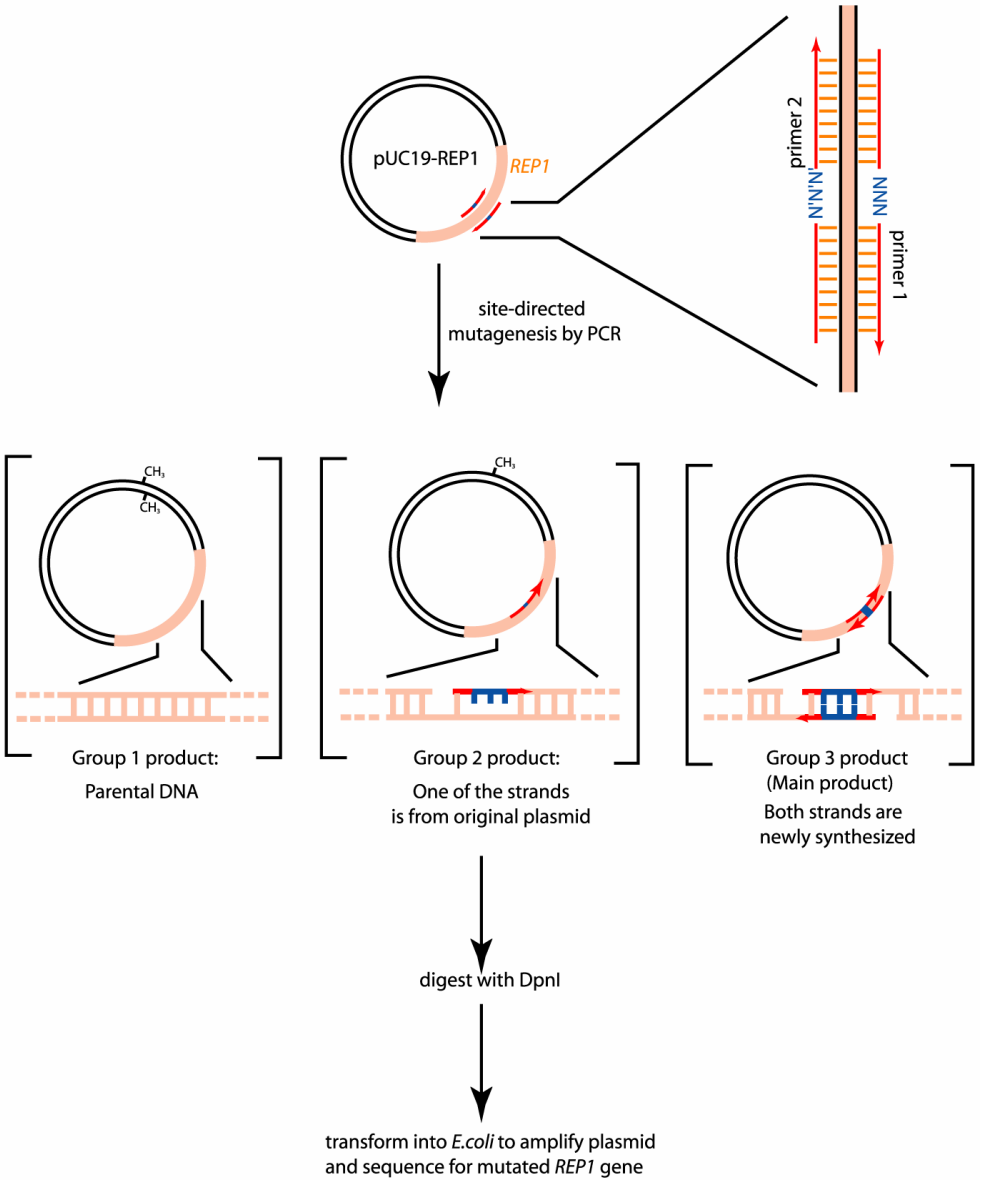


Figure 2.1 Scheme for site-directed mutagenesis. The *REP1* gene templated for mutagenesis is indicated in orange color. The two mutagenic primers used in PCR contain randomized nucleotides at one triplet position (shown in blue color). The template plasmid DNA was prepared from DH5a which is *Dam*$^{+}$: therefore both of its strands were methylated, and the plasmid was sensitive to DpnI restriction enzyme. DpnI digestion would then get rid of the original template plasmid and increase mutagenesis efficiency.

will result in the recruitment of a transcriptional activation domain to the promoter of a reporter gene cassette, and result in its expression. The systems differ, however, in the particular reporter cassettes employed and in the promoters used to express the 'bait' and 'prey' proteins tested. In screening for Rep1p/Rep2p interactors, a cDNA-activation domain fusion library from yeast was used. In this case, the procedure of Finley and Brent (1996) was followed. The positive candidates were subcloned into *E. coli* and sequenced.

2.13 Yeast monohybrid assay (Chapters 4, 5)

The monohybrid assay was done according to the protocols provided by Clontech Laboratories. An approximately 375-bp fragment from the 2 micron plasmid spanning the *STB* locus was amplified by PCR and cloned upstream of the basal promoter of the *HIS3* reporter gene. This transcriptional cassette was integrated into the chromosomal *HIS3* locus. A test protein containing a transcriptional activation domain fusion would be able to elevate *HIS3* transcription if it were able to interact with *STB*. The high level transcription was monitored by resistance to titrated amounts of the His3p-specific inhibitor 3-AT.

2.14 Chromatin immunoprecipitation (Chapter 5)

ChIP assays were carried out as described in Saitoh et al., 1997 with some modifications as described in Kang et al. (2001). A schematic representation of the principle of the methodology is outlined in Fig. 2.2. DNA binding proteins are fixed on the chromatin by chemical cross-linking with formaldehyde. The DNA is sheared to a desired average length (normally in the 300 to 500 bp range) by sonication and immunoprecipitated with antibodies to a given protein. The crosslinks are reversed from the precipitated DNA, and specific sequences are probed by PCR using synthetic oligo primers.

2.15 Plasmid stability assay for testing the functionality of Rep1p variants obtained by site-directed mutagenesis (Chapter 4)

The stability assays were performed in a [cir^0] host harboring a reporter plasmid cp22 (pSTB-REP2 containing the *LEU2* and *ADE2* markers; Table 2.2) as follows. Purified colonies of the [cir^0] strain transformed with the reporter plasmid and the expression plasmid for wild type Rep1p or each of the variant Rep1ps were maintained on minus Ura, minus Leu/dextrose or minus Ura, minus leu/galactose plates. In the expression plasmids, the selectable marker was *URA3*, and the wild type *REP* or variant *rep* loci were placed under the control of the inducible *GAL10* promoter. Single colonies from the glucose and galactose master

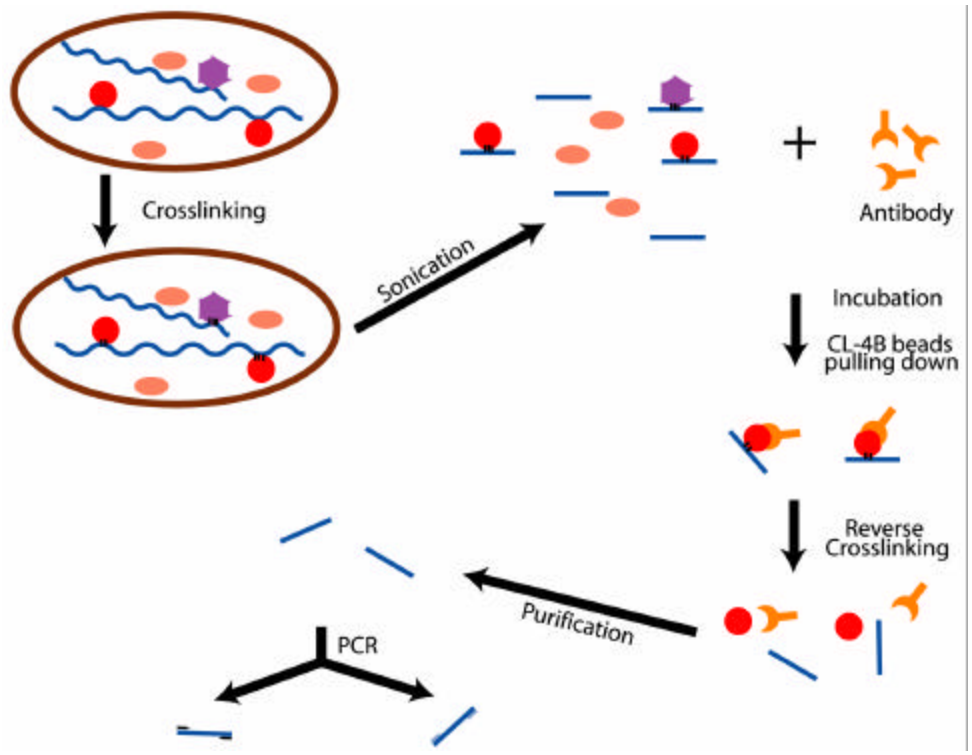


Figure 2.2 A brief outline of the ChIP assay. After chemical crosslinking of chromatin bound proteins to DNA, yeast cells are sonicated to fragment the DNA into short pieces. Specific antibody is added to the cell extract to immuno-pull-down the interested protein along with the DNA fragments crosslinked to it. The protein-DNA crosslinking is then reversed. Finally, PCR is carried out using specific primers to detect suspected DNA sequences in the immunoprecipitate.

plates were spread out on YEP-Dex and YEP-Gal plates, respectively, and grown for three days at 30ºC. The red and white colonies on each plate were counted. Sectored colonies were grouped with the white colonies if the sector size was smaller than one-fourth the colony size, and with the red colonies if the sector size was larger. The plasmid stability index (SI) was then expressed as the ratio of the white colonies to the sum of the white plus red colonies multiplied by 100. The values of SI listed in Chapter 4 are for transfer of colonies from selective galactose plates to YEP-Gal plates.

The potential negative dominance of certain Rep1p mutants was tested by using a similar plasmid stability assay as that described above. The mutant proteins were expressed from the *GAL10* promoter. The test plasmids used were 2 micron circle derivatives containing the *ADE2* marker inserted in the intragenic region between the *REP1* and *RAF1* loci or within the *FLP* locus (2μ-ADE2 and 2μ-ADE2(flp), respectively, listed in Table 2.2).

2.16. Variations of the assays for mitotic stability of plasmids (Chapters 5, 6)

Yeast strains [cir^0] or [cir^+] carrying appropriate mutations were transformed with a test plasmid alone or co-transformed with additional plasmids, as required, to provide desired protein products *in trans*. Purified colonies of the yeast transformants were patched on proper drop-out plates selecting for all the

plasmids, and allowed to grow for two days. Cells from the fresh colonies were transfered using sterile toothpicks into sterile deionized water. Cell suspensions were diluted appropriately, and equal volumes were spread on plates selecting for all plasmids in one case (A) and selecting for plasmids other than the test plasmid in the second case (B). Mitotic stability of the test plasmid was expressed as the number of colonies on plate A divided by the number of colonies on the corresponding plate B.

CHAPTER 3

Evidence for a Potential Connection between the 2 Micron Plasmid and Chromosome Segregation Pathways

3.1 Abstract

1. There is near equivalence between the chromosome and the 2 micron plasmid in the timing of their movement across the cell during the yeast cell cycle and their segregation into mother and daughter cells. This chromosome-like behavior is determined by the Rep/*STB* partitioning system.

2. The Rep1 and Rep2 proteins form a tight complex with the 2 micron plasmid inside the yeast nucleus. This protein DNA association is the basis for the compact clustering of the multiple copies of the plasmid. They appear to remain clustered throughout the cell cycle, and can be detected in yeast chromosome spreads. The plasmid cluster appears to constitute the partitioning entity.

3. A number of distinct mutations that result in inappropriate segregation of chromosomes result in missegregation of the 2 micron plasmids as well. More importantly, the plasmids and chromosomes tend to missegregate in tandem.

4. The plasmid cluster localizes at or near the spindle pole in the vast majority of the cells. Depolymerization of the spindle by treatment with

nocodazole adversely affects the cohesiveness of the cluster, and results in a wider dispersal of the Rep proteins in the yeast nucleus.

3.2 Background

The successful propagation of the 2 micron circle is accomplished via a partitioning system and an amplification system (reviewed in section 1.3.2 of Chapter 1). Two plasmid-coded proteins, Rep1p and Rep2p, in conjunction with a *cis* acting locus *STB* (also called *REP3*) contribute to the partitioning function (Jayaram et al., 1983; Kikuchi, 1983). In addition, host encoded factors may also play a direct or indirect role in plasmid stability (Mehta et al., 2002; Velmurugan et al., 1998; Velmurugan et al., 2000; Wong et al., 2002). Recent *in vivo* and *in vitro* analyses have demonstrated that Rep1 and Rep2 proteins are nuclear localized, exhibit self- and cross-associations, and bind to the *STB* locus. It is suspected that the association between the 2 micron plasmid and the Rep proteins requires the mediation of one or more host factors. *In vitro* studies have demonstrated that urea-solubilized yeast extracts expressing Rep1p and Rep2p or [cir^0] extracts supplemented exogenously with Rep1p and Rep2p can bind *STB* DNA (Hadfield et al., 1995).

Yeast plasmids containing chromosomal *ARS* elements but lacking the 2 micron circle partitioning system have a propensity to be retained in the mother cell during division (Murray and Szostak, 1983; Zakian and Scott, 1982). This mother-daughter bias accounts for their high instability during non-selective propagation of the host cells. The stability of the native 2 micron plasmid

(approximately one plasmid free cell in 10^4 to 10^5 cells per generation) implies that the Rep/*STB* system is able to overcome the segregation bias by one of two plausible mechanisms. Either the plasmids are freed from attachment sites and rendered freely diffusible, or they are actively partitioned or attached to a cellular entity that divides equally between mother and daughter. Currently available evidence can not unambiguously distinguish between the two mechanisms.

The 2 micron plasmid molecules are resident in the nucleus in minichromatin form with standard nucleosome phasing (Livingston, 1977; Livingston and Hahne, 1979; Nelson and Fangman, 1979; Taketo et al., 1980). In addition, their replication by the cellular replication machine, as already indicated, follows normal cell cycle controls. It seems plausible then that the 2 micron circle might also depend on the chromosomal segregation apparatus for its stable inheritance. In this chapter, we have explored the possible connection between plasmid and chromosome segregation using primarily cell biological approaches.

3.3 Results

3.3.1 Direct visualization of a 2 micron-derived plasmid: comparison to a yeast chromosome or a centromeric plasmid

In order to visualize plasmids in live yeast cells, we have utilized the recognition between multiple copies of the lac operator sequence harbored by the

reporter plasmid constructs and a fluorescent version of the lac repressor (by fusion to GFP) expressed from an inducible promoter (Robinett et al., 1996). Cells harboring the marked plasmids were examined by conventional (right panel in Fig. 3.1) or confocal (left panel in Fig. 3.1) microscopy. In a population of [cir^+] yeast cells growing exponentially in selective media, or in cells synchronized in the G1 phase with α-factor, the fluorescently labeled 2 micron derived test plasmid pSV1 was seen most often as a tetrad cluster within the nucleus (>50% of the time). The results shown in Fig. 3.1 were obtained with α-factor-treated cells. Consistent with the nuclear residence of the 2 micron circle (as is the case for *ARS* plasmids as well), the green plasmid fluorescence (from GFP-lac repressor) coincided with the blue nuclear fluorescence (from DAPI; data not shown). At the α-factor concentration used in these experiments (7μg/ml; in SD medium containing required supplements), nearly all the cells showed an unbudded morphology; however, they did not show the typical 'shmoo' phenotype associated with G1 arrest. Nevertheless, they did not progress through the cell cycle unless they were washed free of α-factor. (The same concentration of α factor in rich medium induced shmooing.)

Examination of a large sample of cells revealed occasional deviations from the tetrad pattern of plasmid distribution. In approximately 20% of the plasmid-containing cells, the clusters consisted of triad or diad patterns (Fig. 3.1B

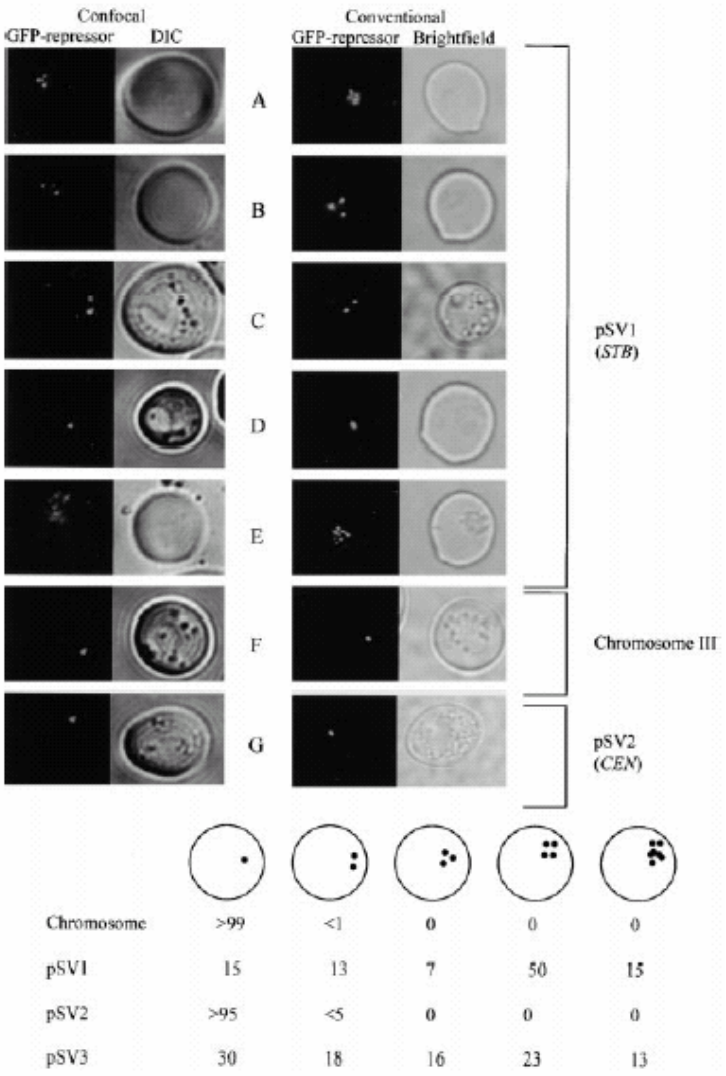


Figure 3.1 Organization and distribution of a 2 micron circle based plasmid pSV1 in a [cir$^+$] yeast strain. The pSV1, pSV2 (*CEN* plasmid) and chromosome III were visualized by the binding of GFP-lac repressor to lac operator sequences contained by the plasmids or by chromosome III. The characteristic forms of the plasmids or that of chromosome III in a G1-arrested [cir$^+$] cell population, as observed by the green fluorescence, are arranged in rows A-G. The patterns of distribution of fluorescent dots for the marked chromosome or the different test plasmids (pSV3 is an *ARS* plasmid without a centromere or *STB*) in a G1 cell population obtained by a-factor arrest are tabulated at the bottom.

and C, respectively), whereas, in approximately 15% of the cells, single fluorescent dots were observed (Fig. 3.1D). Occasionally (15% or less), the plasmid foci were constituted by more than four dots (Fig. 3.1E). In comparison, a marked yeast chromosome appeared as a single fluorescent dot in over 99% of the cells examined (Fig. 3.1F). Similarly, a centromeric plasmid, pSV2, was also detected as a single fluorescent spot in >95% of the cells (Fig. 3.1G), with an occasional cell revealing two fluorescent dots (presumably representing two plasmid copies). By contrast, a population of cells grown selectively for the plasmid pSV3, containing a chromosomally derived replication origin (*ARS*) and none of the components of the 2 micron circle stability system, showed an essentially random distribution of cells containing 1 to 4, and occasionally >4 fluorescent dots. The patterns of plasmid distribution and the frequencies of their occurrence in a G1-arrested cell population are summarized at the bottom of Fig. 3.1.

3.3.2 Kinetics of plasmid segregation during the cell cycle compared to a tagged chromosome

The [cir^+] host strain containing the 2 micron test plasmid pSV1 or the tagged chromosome III was synchronized in G1 phase using α-factor. Following release from α-factor arrest, the plasmid or the chromosome was visualized at

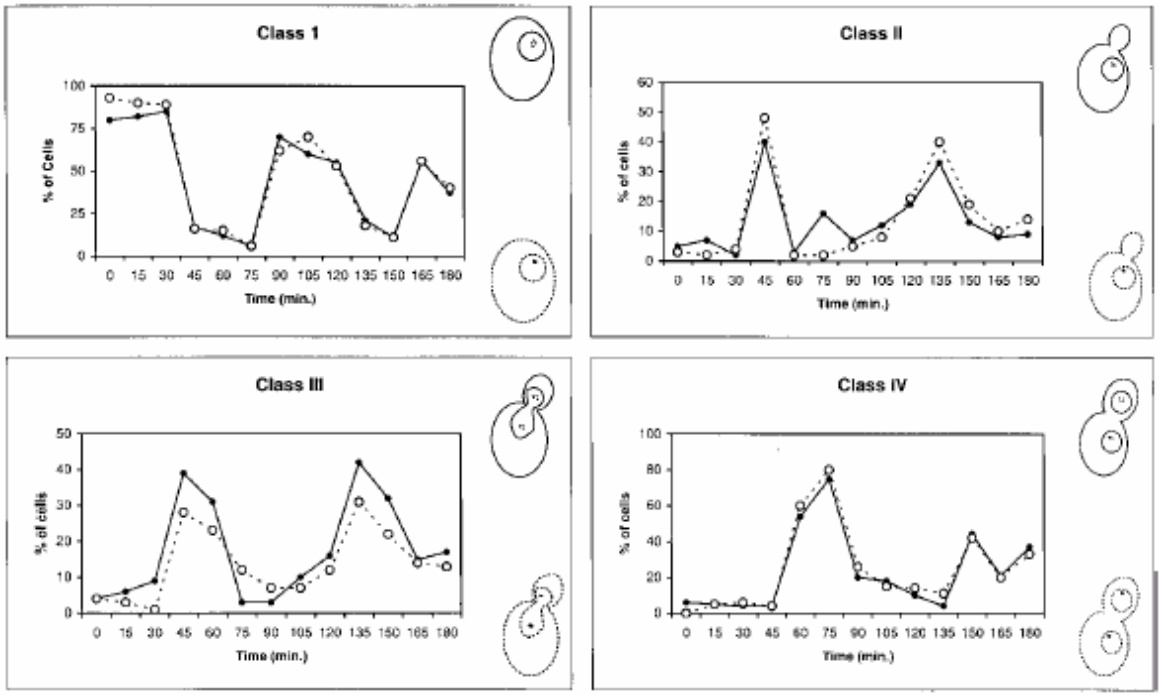


Figure 3.2 Kinetics of segregation of the 2 micron circle based pSV1 plasmid and chromosome III with respect to the cell cycle stages. The host strain harboring pSV1 was released from a-factor induced G1 arrest, and the cell types I-IV (diagrammed at top right corner) were scored over a period of 3 hours. The results were graphed as solid lines connecting the filled circles. An equivalent plot for cells harboring the fluorescent chromosome III is shown by the dashed lines connecting the open circles. Cell types I-IV for the chromosome are schematically represented at the bottom right corner.

various times during cell cycle progression. These observations were quantitated as follows.

We divided the cell population into four classes (I-IV; see the schematic representation in Fig. 3.2), with respect to their progression through the cell cycle from G1 to late G2/M. In order to do this with confidence, we first examined, at different time intervals after release from a factor arrest, a large number of cells for their bud size, their nuclear organization (by DAPI) and their microtubule organization (by immunostaining with antibodies to yeast tubulin). After satisfying ourselves that classes I-IV could be reliably identified by the bud size and nuclear morphology, we proceeded to monitor the localization patterns of pSV1 or chromosome III in cells growing nearly synchronously (Fig. 3.2). The important result from the plots in Fig. 3.2 is that the distribution of pSV1 between mother and bud as a function of cell cycle progression (Fig. 3.2, solid line) was nearly superimposable with a similar plot for chromosome III (Fig. 3.2, dashed line). By contrast, such a neatly overlapping profile with the chromosome was not observed in the case of an *ARS*-plasmid (data not shown).

The above results suggest that chromosome segregation and 2 micron plasmid segregation occur as nearly concurrent events during the yeast cell cycle, at least within the limits of the kinetic resolution of our assays. It is possible that the two processes are mechanistically completely distinct, the observed

coordination between them being merely coincidental. Alternatively, the shared timing suggests that the plasmid might segregate in association with the chromosome or utilize at least parts of the chromosomal segregation machinery for its own dispersal.

3.3.3 Colocalization of the plasmid foci and the Rep1 and Rep2 proteins within the yeast nucleus by immunostaining.

The similarity between the timing of pSV1 and chromosomal partitioning during the cell cycle (Fig. 3.2) raises the intriguing possibility that the Rep/*STB* system might be involved in coupling plasmid and chromosomal segregation machineries. The nuclear localization of the Rep proteins and their *in vivo* interactions (with each other and with the *STB* DNA) revealed by mono- and dihybrid assays are consistent with such a role (Ahn et al., 1997; Velmurugan et al., 1998).

To reveal the localization of the 2 micron plasmid relative to the Rep proteins in yeast cells, immunostaining was employed using mildly fixed cells (Fig. 3.3, row A and B). The Rep proteins were localized by fluorescein-conjugated secondary antibodies and the 2 micron circle derived pSV1 plasmid by Texas red-conjugated secondary antibodies (to lac-repressor antibodies). The red and green fluorescence could be overlaid on each other in >85% of the cells, and occupied a sublocale within the DAPI-staining region. This strong tendency for

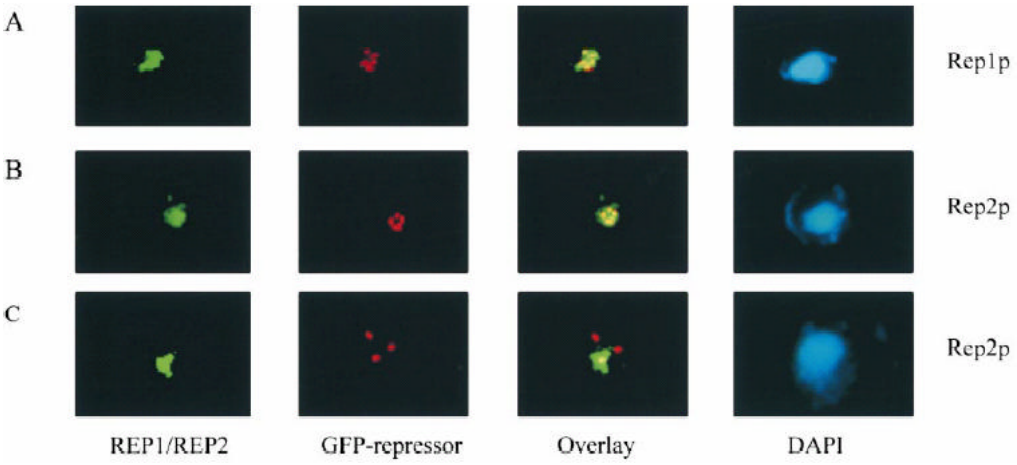


Figure 3.3 Colocalization of Rep1p and Rep2p with the 2 micron derived pSV1 plasmid. Rep1 and Rep2 proteins were expressed from endogenous 2 micron plasmid and visualized by fluorescein-conjugated secondary antibodies. Plasmid pSV1 (row A and B) or pSV3 (row C) was localized using Texas red-conjugated secondary antibodies to lac repressor antibody.

colocalization was absent in the case of the *ARS*-containing pSV3 plasmid and Rep2p (Fig. 3.3, row C) or Rep1p (data not shown). We observed that pSV3 dots were not coincident with the Rep proteins in more than 50% of the cells. These findings agree with the in vivo and in vitro evidence for Rep-*STB* interaction (Hadfield et al., 1995; Velmurugan et al., 1998), and would be consistent with a mechanism by which these proteins might facilitate the docking of plasmid DNA to chromosomes or to some cellular entity that is divided evenly between mother and daughter cells at cytokinesis.

3.3.4 Localization of the Rep1 and Rep2 proteins in chromosome spreads

The observed temporal (and perhaps spatial as well) coupling between chromosome and 2 micron plasmid segregation, and the lack of it in the case of an *ARS* plasmid, would imply that the Rep-*STB* system is the likely coupling agent. In order to probe the potential association (be it direct or indirect) between plasmid and chromosomes, we have followed the localization of the Rep proteins and a 2 micron reporter plasmid in yeast chromosome spreads (Fig. 3.4). Only the data for Rep1p are shown; results with Rep2p were essentially identical.

In [cir^+] spreads, both Rep1p (green) and the plasmid DNA (red) were localized with the chromosomes in exponentially growing (Fig. 3.4A) as well as G1-arrested cells (Fig. 3.4B). As was observed previously (Velmurugan et al.,

2000), plasmids were confined entirely to the Rep1p zone. As a control, we also monitored the Mcd1/Scc1 protein, a subunit of the yeast cohesin complex (Michaelis et al., 1997), in the chromosome spreads. Mcd1p binds along the entire length of chromosomes, although discontinuously, to establish sister chromatid cohesion (Blat and Kleckner, 1999; Laloraya et al., 2000; Tanaka et al., 1999). The Rep1p foci were smaller and sharper relative to the more spread out pattern obtained with the Mcd1/Scc1 protein (Fig. 3.4C), The observed profiles suggest either a more restricted set of chromosome association sites for the Rep proteins or, alternatively, the overlap or at least close proximity between nuclear locales occupied by the plasmid and subchromosomal regions. The low resolution of the chromosome spread assay can not distinguish between these two possibilities. The reporter plasmid present in a [cir^0] strain could not be detected in the chromosome spreads, suggesting that its characteristic localization in the nucleus is mediated by the Rep proteins (Fig. 3.4D, see also E to H). Consistent with the stage-specific expression and binding of the Mcd1 protein to the chromosomes during the cell cycle, Mcd1p-chromosome association was absent in the G1 arrested cells (Fig. 3.4B) and a subset of the cells from the log phase population (Fig. 3.4D). When Rep1p or Rep2p was expressed individually in a [cir^0] strain, no chromosomal association of either protein was observed (Fig. 3.4E and F). When the two proteins were simultaneously expressed in the absence of a resident *STB*

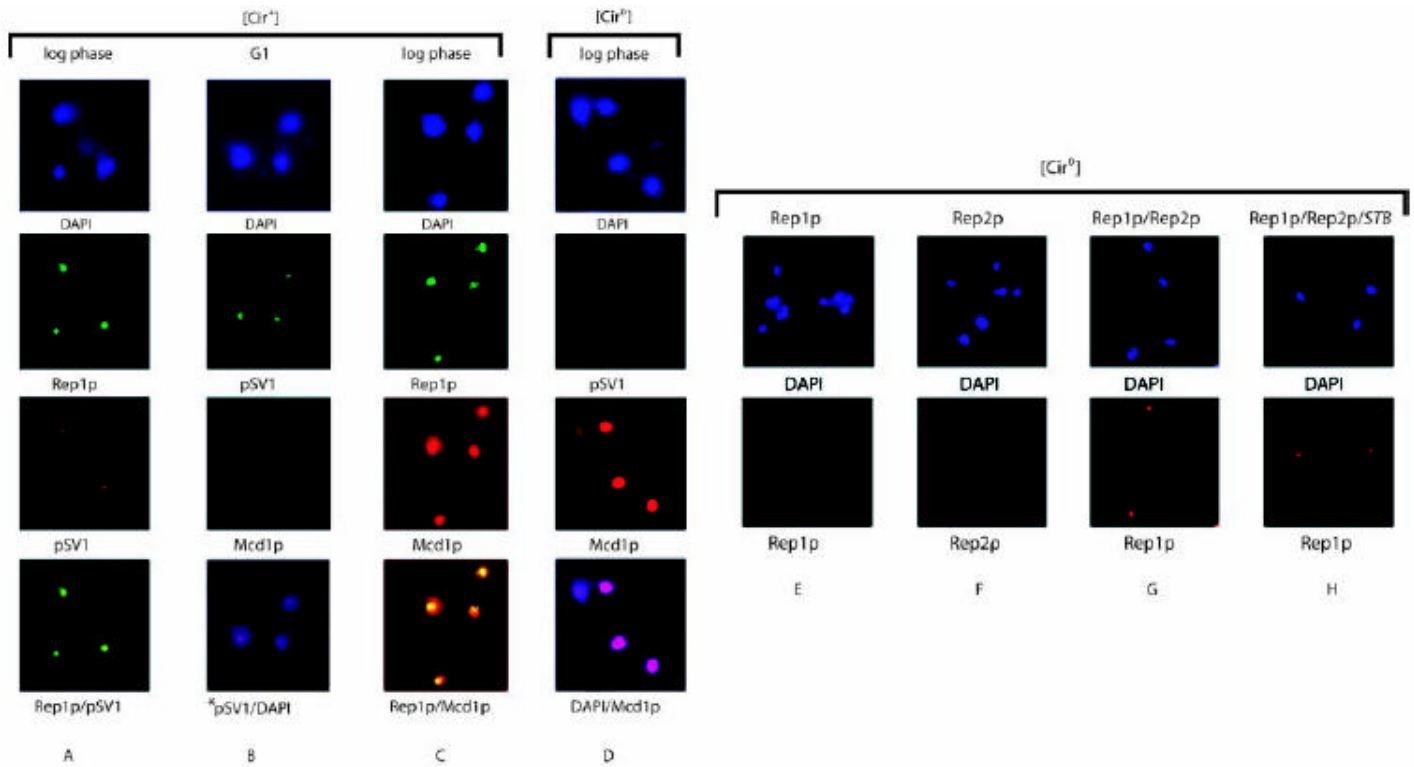


Figure 3.4 Localization of the 2 micron plasmid, the Rep proteins, and the Mcd1 protein in yeast chromosome spreads. Chromosomes were visualized by DAPI, and proteins by immunofluorescence from fluorescein or Texas red-conjugated secondary antibodies. The asterisk on the reporter plasmid pSV1 (bottom panel of B) denotes that the plasmid fluorescence was artificially changed from green to red using Adobe Photoshop software for the purpose of overlaying it on the DAPI fluorescence.

containing plasmid (Fig. 3.4G) or the presence of one (Fig. 3.4H), colocalization of Rep1p with the chromosomes was evident.

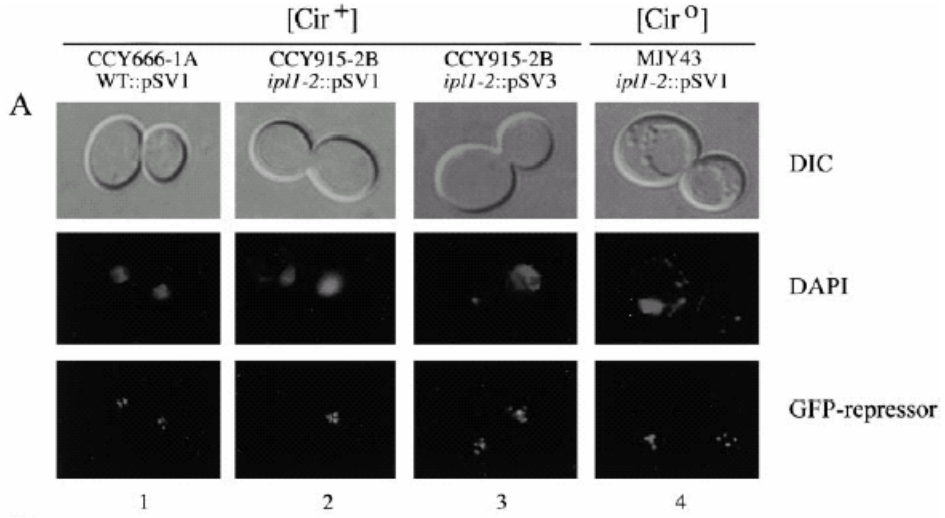
The tight association of the plasmid with Rep1p and Rep2p and the requirement of both proteins for their colocalization with the chromosomes suggest that this process is functionally relevant to plasmid partitioning. The plasmid and Rep protein patterns are independent of the yeast cohesin complex as they show no difference between exponentially growing and G1-arrested cells. Since the Rep proteins bind to the *STB* locus, they may act as match-makers in the potential association between the 2 micron plasmid and the chromosomes.

3.3.5 The 2 micron plasmid tends to missegregate with the bulk of the chromosomes in the *ipl1-2* mutant which is defective for chromosome segregation

To further verify the suspected coupling between chromosomal and 2 micron plasmid segregation, we have examined the partitioning of the plasmids pSV1 (containing the 2 micron circle replication origin and *STB*) and pSV3 (*ARS*-based and lacking *STB*) in a host strain harboring the Ts^- *ipl1-2* mutation. The product of the *IPL1* gene is essential for proper chromosome segregation (Biggins et al., 1999; Chan and Botstein, 1993; Francisco and Chan, 1994; Kim et al., 1999). When shifted to the non-permissive temperature, the predominant fraction of *ipl1-2* cells exhibits a severe chromosome missegregation phenotype.

In the experiments depicted in Fig. 3.5A, chromosomes were identified by DAPI staining and the pSV1 (2 micron circle derived) and pSV3 (*ARS* derived) plasmids by GFP-repressor fluorescence. Unlike the normal segregation observed in the [cir$^+$] wild type host at 37^{o}C (Fig. 3.5A, column 1), the bulk of the chromosomes, along with pSV1, was stuck within the mother or daughter compartment in most large-budded cells from the [cir$^+$] *ipl1-2* host (Fig. 3.5A, column 2). It is known that the *ipl1-2* mutation does not impart a mother/daughter bias in chromosome missegregation (Biggins et al., 1999; Kim et al., 1999).

In Fig. 3.5B, the normal chromosome and plasmid segregation represented by cells of the type '**a**' was contrasted by four types of missegregation represented by types '**b**-**e**'. In **b** and **e**, the DAPI fluorescence was completely excluded from one of the two cell compartments. In **c** and **d**, the fluorescence partitioning was strongly (though not absolutely) biased: approximately 90 to 10 in **c** and 80 to 20 in **d**. While chromosome segregation and plasmid partitioning (indicated by the green fluorescent dots) were tightly coupled in cell types **b**-**d**, they were strongly uncoupled in **e**. The correlation between pSV1 and chromosome locations during missegregation events was nearly perfect in the *ipl1-2* [cir$^+$] host strain (only 4% of type **e** cells in row 2 of Fig. 3.5B). In sharp contrast, the segregation of the *ARS*-based pSV3 in the same *ipl1-2* host was not coupled to chromosome segregation (Fig. 3.5A, column 3; 55% type **e** cells in row 4 of Fig. 3.5B).



B

Strain	plasmid	a	b	c	d	e
CCY666-1A (WT [Cir+])	pSV1	>98%	<1%	<1%	<1%	<1
CCY915-2B (*ipl1-2* [Cir+])	pSV1	23	40	25	8	4
MJY43 (*ipl1-2* [Cir°])	pSV1	19	12	6	2	61
CCY915-2B (*ipl1-2* [Cir+])	pSV3	24	11	6	4	55

Figure 3.5 Chromosome and plasmid missegregation patterns in an *ipl1-2* mutant strain. A. The 2 micron derived pSV1 plasmid or the *ARS*-based pSV3 plasmid was visualized by green fluorescence from the bound GFP-lac repressor, and chromosomes by blue fluorescence from bound DAPI. The *ipl1-2* cells were shifted to 37ºC for 4 hours before they were examined by microscopy. B. the plasmid and chromosome segregation data derived by screening 300-400 large-budded cells in each assay are tabulated. In the schematic diagrams of the cells (a-e), the DAPI staining regions and the fluorescent plasmid dots are indicated. The cells in column **a** represent normal segregation, while those in **b-d** denote chromosome and plasmid missegregation in tandem. The cells in column e typify plasmid segregation uncoupled from chromosome segregation.

A similar degree of uncoupling was also observed for pSV1 in an isogenic but [cir^0] *ipl1-2* host (Fig. 3.5A, column 4; 61% type **e** cells in row 4 of Fig. 3.5B).

The sum of the above results shows that the 2 micron plasmid almost always missegregates with the majority of the chromosomes in *ipl1-2* mutant. And this tandem missegregation depends on the presence of *STB* on the plasmid, and the expression of the Rep proteins.

3.3.6 Missegregation patterns of the 2 micron plasmid in yeast mutants other than the *ipl1* mutant

If the alleged coordination between chromosome segregation and plasmid partitioning suggested by the data from the *ipl1-2* strain is valid, it is quite likely that most (if not all) cellular events that affect chromosome segregation will also affect plasmid segregation similarly. We have therefore followed the effects of several mutations that affect fidelity of chromosome transmission on the behavior of 2 micron-derived plasmids. We describe below the results from mutations in the genes *CTF7*, *CTF13*, *CTF14*/*NDC10* and *NDC80*. These mutations impair chromosome segregation by affecting the association between replicated sister chromosomes via the cohesin complex or by interfering with kinetochore organization and function (explained in more detail under 'Discussion').

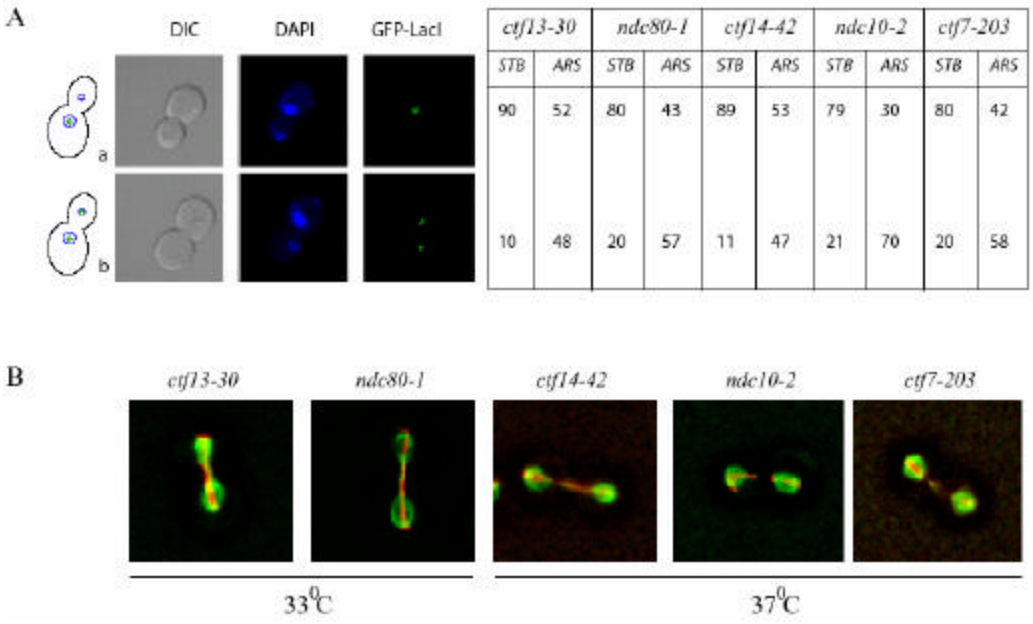
For each mutant strain, logarithmically growing cells were incubated at the non-permissive temperature for 3 hrs, and chromosomes and reporter plasmids

were monitored in large-budded cells (Fig. 3.6A). Cells showing pronounced missegregation of chromosomes, as judged by large inequity in DAPI staining, constituted nearly 70-80% of the population. They were divided into two subgroups: those containing the reporter plasmid in one compartment alone (**a**) or in both compartments (**b**). For simplicity, the differences in the numbers of fluorescent plasmid dots between the two compartments of the class **b** cells are not tabulated here but can be seen in Velmurugan et al. (2000). When plasmids did segregate, the equal distribution patterns (4:4, 3:3 etc.; Velmurugan et al., 2000) far outnumbered the unequal pattern (4:3/2/1, 3:2/1 etc.).

As seen earlier with the *ipl-1*, there was a striking correlation between the tandem missegregation of the chromosomes and the 2 micron-derived plasmid in all mutant strains at the non-permissive temperature (Fig. 3.6A). The presence of the plasmid in a compartment lacking chromosomes was seen in at most 21% of the cells examined. In contrast, the *ARS* plasmid was found in the chromosome-free compartment in roughly 50% of the cells for four of the mutants and 70% of the cells for the fifth mutant (*ndc10-1*). In large-budded cells from a wild type strain grown at 30^0C or 37^0C or from the mutant strains grown at 30^0C, the 2 micron plasmid was almost always present in both cell compartments (data not shown). The near equivalence in DAPI staining in these compartments indicated normal chromosome segregation.

We wanted to rule out the possibility that nuclear elongation was not affected by the non-permissive temperature (Fig. 3.6B). If this were the case, plasmids might give the misimpression of staying with the chromosomes only because they are nuclear resident. We were careful to examine plasmids only in those cells where the chromosomal bulk was confined to one cell compartment but at least a trace of DAPI was present in the other. Thus, we were reasonably certain that we were truly scoring missegregation as opposed to non-segregation of chromosomes. In addition, the random segregation observed with the *ARS*-plasmid was also reassuring – since these plasmids are also nuclear localized. We were also concerned whether the mitotic spindle was assembled and arranged normally in the mutant strains. We examined the organization of the spindle and the disposition of the nuclear envelop after the cells were shifted to the non-permisisve temperature. This was accomplished by tagging the nuclear membrane protein Nup49p with CFP and tubulin with YFP (Fig. 3.6B) simultaneously. For each of the mutations, normal nuclear migration and spindle elongation were observed for over 80% of the cells in the population.

Based on the data presented above, we argue that the chromosome and plasmid partitioning pathways either overlap with each other in at least some of their steps or the two are coordinately regulated. For the *ctf14* and *ndc80* mutants, missegregation of the 2 micron test plasmid in tandem with chromosomes is dependent on the Rep proteins. In a [cir^0] mutant background, the 2 micron



	ctf13-30		ndc80-1		ctf14-42		ndc10-2		ctf7-203	
	STB	ARS	STB	ARS	STB	ARS	STB	ARS	STB	ARS
a	90	52	80	43	89	53	79	30	80	42
b	10	48	20	57	11	47	21	70	20	58

Figure 3.6 Partitioning of *STB*-plasmids and *ARS*-plasmids in yeast mutants that are defective in chromosome segregation. A. The temperature-arrested cells were categorized into two types: **a, b**. The representative cells shown here are from the *ctf13-30* strain. The chromosome and plasmid profiles were scored by DAPI and green fluorescence, respectively. The values for each cell type were derived from approximately 450 largely budded cells for each strain. B. The nuclear membrane and the mitotic spindle were visualized by fluorescence microscopy in live yeast cells expressing Nup49p-CFP (Cyan Fluorescent Protein) and Tub1p-YFP (Yellow Fluorescent Protein) simultaneously. Red and green colors (tubulin and nuclear membrane, respectively) were added artificially using the Adobe Photoshop software. The patterns shown here are representative of approximately 80 percent of the cells shifted to the restrictive temperature.

plasmid loses its strong chromosome directed bias at the non-permissive temperature (S. Velmurugan and M. Jayaram, unpublished data). Plasmid segregation in the other mutants has not yet been tested in the [cir^0] background.

3.3.7 Effect of microtubule depolymerization on the integrity of the 2 micron plasmid fluorescent foci

Previous studies from our group have shown that the 2 micron plasmid cluster, viewed indirectly by immunofluorescence from the associated Rep proteins, tends to either overlap with the spindle pole or localizes in close proximity to the pole (Velmurugan et al., 2000). Furthermore, there is a bipolar gradient of Rep protein concentration that decreases steadily from the spindle poles to the midsection of the spindle. Since we suspected that the plasmid and chromosome segregation might be coupled to each other, it was pertinent to ask whether the 2 micron plasmid might utilize the mitotic spindle for its partitioning. If plasmid molecules are tethered to chromosomes, the spindle would, by default, provide the pulling force to dispatch them to opposite cell poles along with pairs of sister chromatids. Alternatively, plasmids may be actively transported by a spindle-associated motor protein. Or, plasmids may utilize the spindle/spindle pole as a compass for directing them to a cellular entity that is equally partitioned between daughter cells. Because of insufficient information to formulate specific testable models at this time, we decided to simply examine the overall

organization of the plasmid cluster (which is the partitioning moiety) and the nuclear localization of the Rep proteins (which are essential for partitioning) following the disassembly of the spindle apparatus.

A [cir$^+$] host strain was treated with nocodazole (20 µg/ml) for 2 hrs such that 80-85% of the cells were arrested in the G2/M phase as judged by microscopy. Immunofluorescence staining for tubulin in these large-budded cells revealed nearly complete disassembly of the mitotic spindle, although a limited amount of residual fluorescence was detectable at some spindle poles (Fig. 3.7, middle panels in rows B and D). Along with the disassembly of the spindle, the Rep proteins showed a less compact, more disperse, pattern of nuclear localization (Fig. 3.7, right most panels in rows B and D). Nocodazole did not affect the steady state levels of the Rep1p or Rep2p, as assayed by Western blot analysis of total yeast cell extracts (data not shown). The normal tubulin and Rep protein patterns (in untreated cells at the G2/M phase) are shown in rows A and C of Fig. 3.7 for reference.

We then performed confocal Z-series sectioning of the yeast nucleus (see 'Materials and Methods') to examine the organization of the 2 micron plasmid clusters following disassembly of the spindle (Fig. 3.7, rows E and F). The assay provides the number of frames within which the GFP-fluorescence associated with the reporter plasmid is confined (the plasmid residence zone; see Fig. 3.7). The smaller this number, the more tightly organized is the plasmid cluster. To

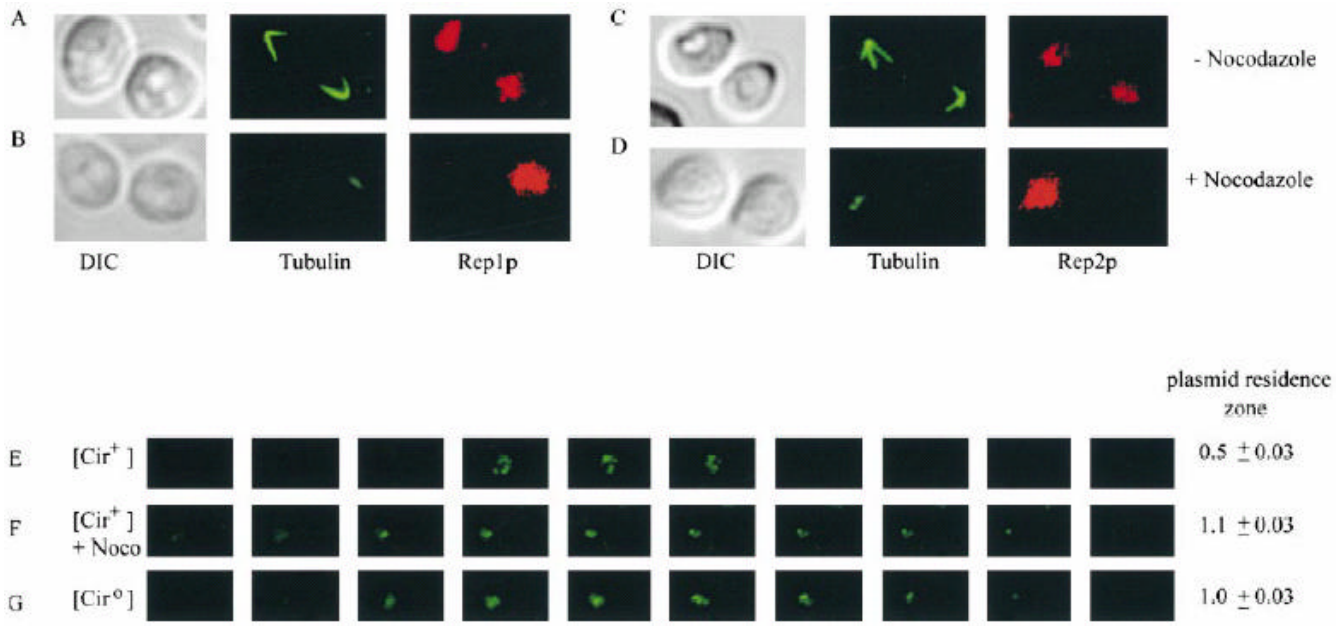


Figure 3.7 Nocodazole treatment alters the pattern of Rep protein and pSV1 plasmid distribution in the nuclei. After a 2-hour treatment of [cir$^+$] cells (CCY666-1A) with nocodazole at 30ºC, the Rep proteins and tubulin were visualized by immunofluorescence using Texas red- and fluorescein-conjugated secondary antibodies, respectively. The results for Rep1p and tubulin localization in the control and nocodazole-treated cells are shown in A and B, respectively. The corresponding results for Rep2p and tubulin are displayed in C and D, respectively. Typical results of scanning the pSV1 plasmid by z-series sectioning using confocal microscopy are presented in E-G. The leftmost frame in each row represents the arbitrarily fixed boundary beyond the edge of plasmid fluorescence. From this start point, a total of 20 consecutive frames (each 0.25 μm) were scanned for each sample. Only ten of these frames, representing every alternate one, are shown here. For each cell examined, a similar z-series scan was performed for the DAPI fluorescence also. The value for the size of the plasmid zone listed at the right of each row is the mean ratio of the green fluorescence range (emitted by GFP-repressor-bound plasmids) to the blue fluorescence range (emitted by DAPI-bound bulk chromosomes). Untreated [cir$^+$] cells are shown in E.

compensate for differences in overall nuclear size, the values were normalized to the range of DAPI fluorescence in the same cells, also estimated by Z-series sectioning. In the control [cir^+] cells, the range of the plasmid zone was 0.5 ± 0.03 (row E of Fig. 3.7). By contrast, this range was nearly doubled in nocodazole treated [cir^+] cells (1.1 ± 0.03; row F of Fig. 3.7) or in untreated, but [cir^0] cells (1.0 ± 0.03; row G of Fig. 3.7). Estimates of cell and nuclear sizes (from scanning DIC and DAPI images, respectively) showed that the cell enlargment or nuclear expansion as a result of nocodazole treatment was no more than 20% (data not shown). Thus, the lack of an intact microtubule array or the absence of a functional Rep system (as in the [cir^0] host) has the common effect of slackening the cohesive forces between plasmid molecules.

3.4. Discussion

3.4.1 Plausible coupling or overlap between plasmid and chromosome segregation pathways

Direct observations of plasmid dynamics during the cell cycle have revealed a striking similarity between the 2 micron plasmid and the chromosomes in their segregation kinetics. Furthermore, the plasmid clusters could be localized to chromosome spreads in both G1-arrested as well as normally cycling cell populations. Both the Rep1 and Rep2 proteins are indispensable for the plasmid to

be detected in the chromosome spreads. Even when an *STB*-containing plasmid is absent in the cell, the co-expressed Rep1 and Rep2 proteins were visible in the chromosome spreads. Conversely, in the absence of either Rep1p or Rep2p, an *STB*-plasmid could not be detected in the chromosome spreads. One simple explanation consistent with these observations is that the Rep1p-Rep2p complex is targeted to certain sites within the chromosomes. Alternatively, the Rep1 and Rep2 proteins may comigrate to locales in the nuclear scaffold that also provide anchoring points for chromosomal domains. Because the Rep proteins exist in association with the 2 micron plasmid molecules, the latter become either tethered to chromosomes or localized in the vicinity of chromosomes by sharing common subnuclear attachment sites. It is possible to envisage how either mechanism could be important in coupling plasmid and chromosome segregation pathways.

Our findings that Rep1 and Rep2 proteins colocalize with the 2 micron plasmid inside the yeast nucleus to form a tight cluster become particularly significant when considered in the light of the chromosome spread data. They suggest that, despite being a multi copy plasmid, the functional 2 micron circle entity is likely a single high-order complex in which the plasmid DNA (presumably the *STB* locus) and the Rep proteins are intimately associated. During our observations of plasmid segregation in continuously dividing cells (by time-lapse fluorescence microscopy; S. Velmurugan and M. Jayaram, unpublished data), we have never seen an obvious declustering of plasmid at any

particular stage of the cell cycle. We therefore believe that the plasmid cluster is the partitioning unit. The effective plasmid copy number in this case is essentially unity, and may explain why the 2 micron circle has evolved an active partitioning system in spite of its apparent high copy number.

If the yeast chromosomes and the 2 micron plasmid are segregated in a coupled manner (we suspect this to be the case based on evidence presented in this and other chapters of this thesis as well as corroborating data that are not presented here), we would argue that the Rep proteins are the best candidates to mediate this coupling process. They could act as match makers by interacting on the one hand with the *STB* DNA (established by this and related studies; Ahn et al., 1997; Velmurugan et al., 1998) and on the other with chromosomes or with 'partitioning centers' within the nucleus (merely a conjecture at this time). We are particularly intrigued by the possibility that the plasmid cluster may in some fashion be associated with the kinetochore complex. Initially, this idea arose from the fact that the plasmid cluster was most often seen to be coincident with the spindle pole or at least remain quite close to it (Velmurugan et al., 2000). This localization pattern is reminiscent of that observed for the kinetochore regions of chromosomes. This notion is consistent with the subsequent finding that the yeast cohesin complex associates with the *STB* locus of the 2 micron plasmid (Chapter 5; Mehta et al., 2002). The centromeric and proximal regions constitute one of the most prominent cohesin loading sites on the chromosomes. A kinetochore

mediated chromosome to plasmid tethering therefore seems eminently plausible. However attempts to detect interactions between some of the protein components of the kinetochore and the Rep proteins have not been successful.

3.4.2 The cellular mitotic machinery may be important for plasmid segregation

The present work has established the propensity of the 2 micron plasmid to co-segregate (as well as co-missegragate) with the chromosomes by using a number of conditional mutations that affect distinct steps of the complex pathway that ensures faithful distributions of replicated chromosomes to daughter cells. The first set of data was obtained with the *ipl1* mutation, and subsequently the results were corroborated with *ctf7, ctf13*, *ctf14*, *ndc10* and *ndc80* mutations.

The Ipl1 protein is a kinase that appears to act in association with the Sli15 protein (a substrate for Ipl1p mediated phosphorylation). Failure of chromosome segregation in the *ipl1-2* mutant cells is often associated with the abnormal distribution of the spindle pole-associated Nuf2 protein (Kim et al., 1999). The Ipl1 kinase has also been shown to function in the binding of kinetochores to the spindle in a bipolar orientation (Biggins et al., 1999; Kang et al., 2001; Tanaka et al., 2002). The products of the *CTF13* and *CTF14/NDC10* genes are integral components of the CBF3 protein complex that binds to the CDEIII element of yeast centromeres (Doheny et al., 1993; Goh and Kilmartin, 1993; Jiang and

Carbon, 1993; Jiang et al., 1993; Strunnikov et al., 1995), and is required for the association of centromeres with the yeast cohesin complex (Russell et al., 1999; Tanaka et al., 1999). The Ndc80 protein is part of a kinetochore associated complex (Janke et al., 2001; Wigge et al., 1998; Wigge and Kilmartin, 2001). The Ctf7 protein is important for the establishment of cohesion between sister chromatids but is not itself included in the cohesin complex (Skibbens et al., 1999; Toth et al., 1999). Nonfunctionality in any one of these proteins results in impaired partitioning of chromosomes. And as revealed by the present studies, similar effects are manifested in 2 micron plasmid segregation as well.

The findings from this study suggest that the integrity of the yeast mitotic spindle may be important for the partitioning of the 2 micron plasmid. First, the plasmid cluster resides at or near the spindle pole. Second, upon nocodazole treatment and depolymerization of the spindle, the Rep1 and Rep2 proteins show a rather dispersed pattern of localization in contrast to their normally compact organization. Third, concomitant with the altered Rep protein profiles, the tightness of the plasmid cluster is also relaxed, as evidenced by the expanded plasmid residence zone revealed by Z-series sectioning. The plasmid clusters now become more or less identical to those formed by *ARS* plasmids (lacking *STB*) or by *STB*-containing plasmids when they are deprived of one or both of the Rep proteins (in a [cir^0] host strain). Admittedly, the role of the spindle in 2 micron circle partitioning is highly speculative at present. However, as will be described

in Chapter 5, the recruitment of the cohesin complex (central to chromosome segregation and possibly relevant to plasmid segregation) to the *STB* locus can occur only when the nuclear microtubules are intact. Spindle disassembly by nocodazole causes the bound cohesin complex to dissociate from *STB*. However, removal of the drug and restoration of the spindle results in reassociation of cohesin with *STB*.

3.4.3 Unanswered questions

While we have used a variety of cell biological and molecular genetic strategies to implicate a possible connection between chromosome and 2 micron circle partitioning mechanisms in yeast, this connection is quite tenuous at the moment. One fundamental question is whether we can identify specific conditions other than impairment of the Rep/*STB* system (say those imposed by certain host mutations) that can uncouple plasmid segregation from chromosome segregation? If successful, we may be able to identify steps unique to the plasmid pathway and decipher how these might then feed into the chromosome pathway. So far we have not been able to unveil such conditions.

If the plasmid hitchhikes on the chromosomes to achieve equal segregation, it would be impossible to functionally dissociate the two pathways. However, in this case, we may be able to probe for potential plasmid tethering sites on the chromosomes, based on the assumption that such sites would be target

sequences for binding by one or both of the Rep proteins. Chromosome immunoprecipitation by antiobodies to Rep1p or Rep2p and probing a whole genome array using the immunoprecipitated DNA might reveal the existence of chromosomal regions for plasmid tethering. Indeed, these experiments are now in progress.

CHAPTER 4

Mutagenesis and Characterization of 2 Micron-encoded Rep1p: Interactions with Rep2p and *STB*

4.1 Abstract

1. By site-directed mutagenesis followed by appropriate interaction analyses and plasmid stability assays, we have revealed different classes of Rep1p mutations that are consistent with a model for plasmid partitioning hypothesized from earlier genetic experiments.

2. One unexpected result from this study was the failure of the Rep1p mutants to exhibit negative dominant behavior over wild type Rep1p even when present in excess over the latter. We do not have a satisfactory explanation for this observation. One possibility is that a small fraction of wild type Rep1p present in the presumed Rep1p-Rep2p-*STB* complex is sufficient to elicit normal partitioning. A second possibility is that the ternary complex is stable only when both the Rep1p-Rep2p interactions and the Rep1p-*STB* interactions are normal. This would provide a mechanism for excluding the defective Rep1p mutant (even when present in excess) and incorporating only wild type Rep1p (present in much smaller amounts) into the partitioning complex.

4.2 Background

The 2 micron plasmid is the most well-known and perhaps the most extensively studied extrachromosomal DNA element in *Saccharomyces* yeast. However, several plasmids with similar genetic organization have been found so far in other yeast genera that are not necessarily close relatives of *Saccharomyces* (Chen et al., 1986; Toh-e et al., 1984; Toh-e et al., 1982). One such example is plasmid KD1 isolated from *Kluyveromyces drosophilarum* (Chen et al., 1986); some of the others were discovered in *Zygosaccharomyces* yeasts used in the fermentation of soy sauce (Toh-e et al., 1984; Toh-e et al., 1982). The genomic configurations of these plasmids are depicted in Fig. 4.1, and their protein coding capacities are listed in Table 4.1. All plasmids contain the gene for a site-specific recombinase and one pair of inverted repeats, which divides the plasmid into two unique DNA regions. The target sites for the recombinase are embedded in the repeats, and their sequence can be guessed with confidence from the known features of the target sites for the 2 micron circle Flp recombinase (reviewed by Jayaram et al., 2002) or the R recombinase of the pSR1 plasmid (Araki et al., 1992). The orthologues of the 2 micron Rep1 and Rep2 proteins are also encoded by these plasmids. A site within pSR1 that is required in *cis* for stable propagation (Jearnpipatkul et al., 1987) has been identified. And this site encompasses a series of repeated elements in direct and inverted orientations with a reasonable degree (65-80%) of cross homology. However, whether these repeated elements are

responsible for the stability-enhancing function of the site has not been determined. The conserved structural and functional organization of the plasmids suggests that, analogous to the 2 micron plasmid, they also utilize a partitioning system and an amplification system to ensure their stable maintenance. Consistent with this idea, the locations of plasmid origins (in cases where they have been mapped) show marked asymmetry with respect to the inferred recombination sites. Note that this asymmetry is a basic requirement for replicative amplification according to the model proposed by Futcher (Futcher, 1986; see also Fig. 1.14 in Chapter 1). The coding capacity for a small protein corresponding to the Raf1 protein of the 2 micron circle (made from the open reading frame D and implicated to positively regulate Flp expression) has been identified only in one other plasmid (pSM1).

Among the yeast plasmids, the Flp (site-specific recombinase) orthologues are easily recognized by virtue of the conserved catalytic regions with invariant signature active site residues. Amino acid alignments of the other proteins indicate that the Rep1p orthologues share the highest degree of amino acid homology (Murray et al., 1988). There is only sparse sequence similarity among the Rep2 proteins. The C-terminal portion of the Rep1 protein of the 2 micron plasmid shares sequence similarities with nuclear lamins and vimentin (Wu et al., 1987), and is predicted to form a coiled coil structure. It has been suggested that Rep1p may utilize this structural feature to anchor the plasmid to the nuclear

matrix or scaffold. In Fig. 4.2, the Rep1p sequences from four of the yeast plasmids are aligned to highlight their sequence similarities and identities.

The rationale for the mutational analysis of Rep1p is as follows. According to the current model for partitioning, the interactions between the two Rep proteins and those between the Rep proteins and the *STB* locus are important in 2 micron plasmid maintenance. This model predicts two obvious types of mutations in Rep1p that should result in plasmid instability: those that cause loss of interaction with Rep2p and those that abolish *STB* association. Some loss-of-function mutations might have the dual effect of interfering with both Rep2p and *STB* interactions. The model does not exclude mutations in Rep1p that might affect partitioning at steps other than interactions with Rep2p and *STB*. One would predict that analogous types of mutations in Rep2p should also lead to defective plasmid partitioning.

We chose to initially focus on the Rep1 protein because of the degree of its sequence conservation with the Rep1-like proteins of the other yeast plasmids. The most highly conserved amino acid positions provided the obvious targets for mutagenesis followed by functional characterization. Since Rep1p interacts with itself, it is reasonable to expect that mutations that disrupt this interaction might also lead to plasmid instability. Since the *in vivo* assay for Rep1p self interaction is not very tight, we have not sought this particular class of mutations in the present study.

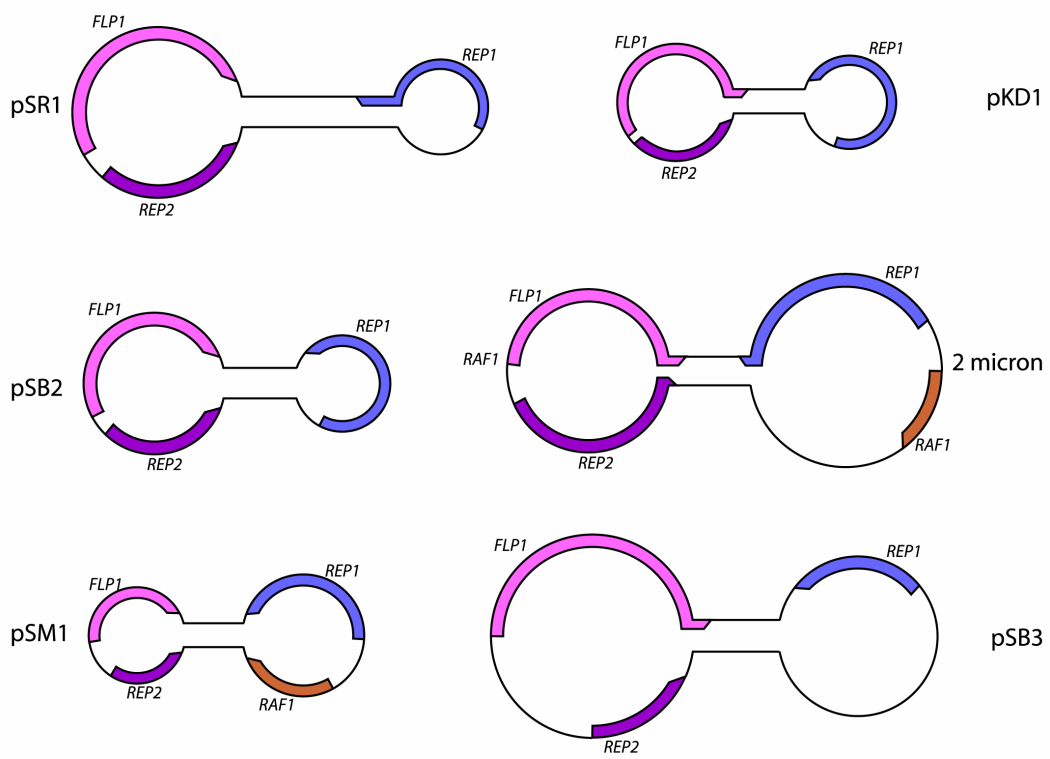


Figure 4.1 Structural organization of 2-micron-circle-like plasmids. The schematic diagrams are drawn approximately to scale and indicate the relative positions of the inverted repeats (horizontal lines) and unique sequences (circular regions). The open reading frames (colored regions, signifying the 5' to 3' orientation from the flush to the slanted end) are also indicated and labeled using 2-micron-circle gene nomenclature. (Adapted from Broach and Volkert, 1991)

Table 4.1 Structural features of 2-micron-circle-like plasmids from yeast

Plasmid	Source[a]	Size (bp)	Open reading frames[b]			
			FLP1	*REP1*	*REP2*	*RAF1*
pSR1	*Z. rouxii*	6251	490	410	233	
pSB1	*Z. bailii*	6550				
pSB2	*Z. bailii*	5415	474	408	158	
pSB3	*Z. rouxii*	6615	568	322	178	
pSM1	*Z. fermentati*	5416	372	394	260	200
pKD1	*K. drosophilarum*	4757	447	415	212	
2-micron	*S. cerevisiae*	6318	387	373	295	180

[a] *(S) Saccharomyces; (Z) Zygosaccharomyces; (K) Kluyveromyces*.
[b] Sizes in amino acid codons of the open reading frames identified by sequence analysis of the plasmids.
(Adapted from Broach and Volkert, 1991)

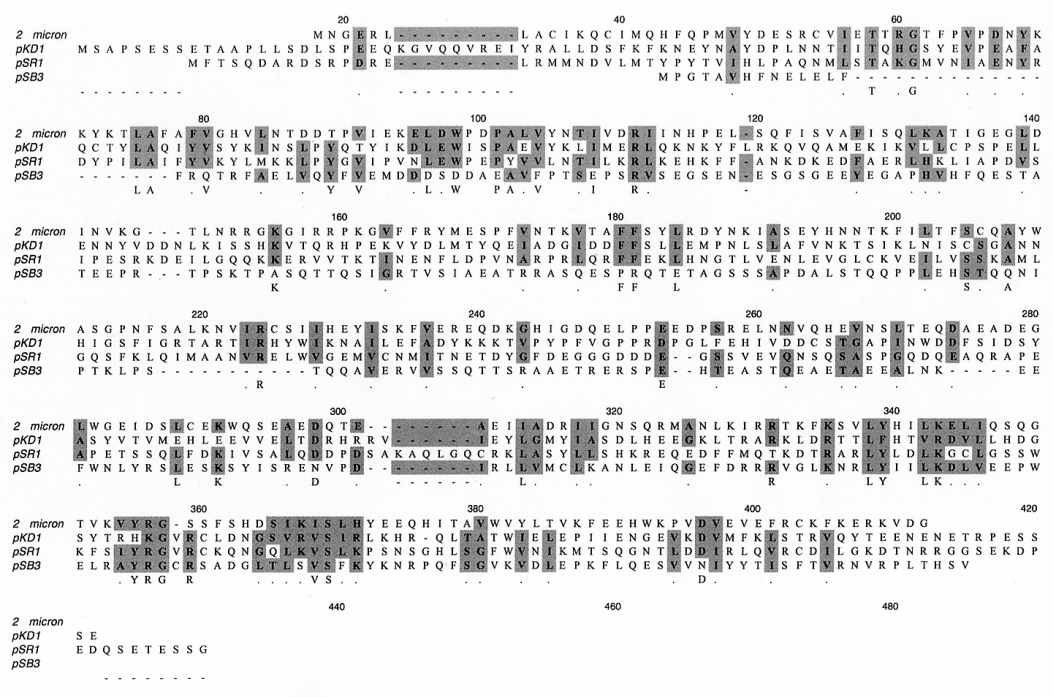


Figure 4.2 Alignment of the Rep1 proteins encoded by the 2 micron circle, pKD1, pSR1 and pSB3. Compared to the Rep1p of pSB3, those of the other plasmids have an extension at the N-terminus. Amino acid positions that are highly conserved are indicated by the shading. When all four or at least three proteins share a common residue at a given position, that residue is indicated at the bottom of the alignment; and in almost all cases, the variant amino acid is chemically related to the conserved one. The shaded areas also comprise residues that have common hydrophobic or hydrophilic properties.

4.3 Results

4.3.1 Site-specific mutagenesis of Rep1 protein by PCR

To mutagenize Rep1p at its conserved residues, we employed a protocol for site-specific mutagenesis by PCR that is described under 'Material and Methods' (chapter 2). Briefly, the *REP1* gene cloned into pUC19 was used as the template for PCR mutagenesis. By using two degenerate oligos as primers, each selected amino acid position was mutated to multiple variant residues. The pool of mutants was transformed into *E. coli*, recovered as individual clones, and each clone was sequenced to determine the nature of a particular mutation.

Using the above procedure, we obtained 148 different mutations in all, corresponding to 24 conserved amino acids in Rep1p (Fig. 4.2). Since this was a relatively large library, we decided to first focus on one or two mutations at each position that caused a chemically distinct or at least non-conservative amino acid substitution. The premise was that such changes are the most likely ones to result in a loss of Rep1 function. The tyrosine substituent at ser-330 was chosen to probe two possibilities: (a) that a primary hydroxyl group at this position might suffice for Rep1 activity; (b) on the other hand, the more bulky aromatic side chain of tyrosine might interfere with activity. Since difficulties were encountered during subcloning of the Rep1p mutants at positions 39 and 257 (V39D and L257E), they were omitted from more detailed functional characterization.

Table 4.2 Summary of mutations generated in Rep1p

W.t. a.a.	Mutant a.a.* (codon)	W.t. a.a.	Mutant a.a.* (codon)	W.t. a.a.	Mutant a.a.* (codon)	W.t. a.a.	Mutant a.a.* (codon)
THR 32	**LYS (AAG)**	LEU 154	ALA (GCC)	ALA 276	SER (TCT)	LYS 305	LEU (CTA)
	GLU (GAG)		GLN (CAG)		PHE (TTT)		PRO (CCT)
	ALA (GCG)		PRO (CCC)		**PRO (CCA)**		**PRO (CCA)**
	GLU (GAG)		GLN (CAA)		PRO (CCT)		SER (TCA)
	SER (TCG)		ALA (GCG)		LEU (CTA)		PHE (TTT)
	ALA (GCC)		GLU (GAG)		THR (ACA)		HIS (CAT)
	ASN (AAC)		SER (TCG)		VAL (GTT)		
	ILE (ATC)		**SER (TCC)**		LEU (TTA)	ILE 308	ASP (GAC)
					THR (ACT)		GLU (GAG)
VAL 39	ALA (GCC)						THR (ACC)
	ASP (GAC)	ASN 166	**LYS (AAA)**				**TYR (TAC)**
	SER (TCC)		ALA (GCA)				ASN (AAC)
	SER (TCG)		THR (ACC)	LYS 297	**GLN (CAA)**		THR (ACG)
	ASN (AAC)		ALA (GCG)		LEU (TTA)		ALA (GCG)
			ALA (GCC)		HIS (CAT)		
TYR 43	VAL (GTG)				SER (TCT)	TYR 317	**ILE (ATC)**
	ALA (GCG)				TYR (TAT)		SER (TCC)
	SER (TCC)	ILE 193	**TYR (TAC)**		PRO (CCT)		THR (ACC)
	THR (ACC)		ASP (GAC)				LEU (TTG)
	ALA (GCC)		THR (ACG)				ASN (AAC)
			ALA (GCG)				GLU (GAG)
ALA 50	SER (AGT)		GLU (GAG)	LEU 300	ALA (GCA)		PHE (TTC)
	ASP (GAT)		SER (TCC)		PRO (CCC)		
	ASN (AAT)				LYS (AAG)		
	SER (TCA)				PRO (CCG)	ARG 318	ALA (GCC)
		GLU 200	ASP (GAC)		GLU (GAG)		ASN (AAC)
PRO 75	HIS (CAT)		THR (ACC)		GLN (CAA)		**ASP (GAC)**
	GLY (GGA)		**ALA (GCC)**		**TYR (TAC)**		ILE (ATC)
	ALA (GCT)		ALA (GCA)				THR (ACC)
	SER (TCT)						TYR (TAC)
	ASP (GAT)						SER (TCC)
	GLY (GGT)	LEU 257	HIS (CAC)	TYR 301	VAL (GTC)		
			ASN (AAC)		SER (TCG)		
VAL 78	SER (TCG)		SER (TCA)		THR (ACG)	GLY 319	ALA (GCC)
	ASN (AAC)		TYR (TAC)		**LEU (TTG)**		SER (AGC)
	TYR (TAC)		ALA (GCG)		ASP (GAC)		**LYS (AAA)**
	ASP (GAC)		GLU (GAA)		THR (ACC)		SER (TCC)
	THR (ACC)		GLN (CAA)				ASN (AAT)
	THR (ACG)						THR (ACA)
	LYS (AAG)						
		GLU 260	THR (ACC)	LEU 304	TYR (TAC)		
GLU 110	THR (ACC)		**TYR (TAC)**		GLU (GAG)	SER 330	ASN (AAC)
	ASN (AAC)		ALA (GCA)		**PRO (CCA)**		THR (ACC)
	SER (TCA)		THR (ACA)		PRO (CCC)		ALA (GCC)
	ALA (GCA)		ASP (GAC)		THR (ACC)		**TYR (TAC)**
	ALA (GCC)		ALA (GCC)		THR (ACA)		CYS(TGC)

* Bold amino acids are the ones selected for further analyses.

4.3.2 Subcellular localization of Rep1p mutants

Since the two Rep proteins are involved in the segregation of the nuclear resident 2 micron plasmid, the nucleus would be the proper functional locale for these proteins. Indeed, results from previous fluorescence microscopy experiment have supported this assumption (Chapter 3; Ahn et al., 1997; Scott-Drew and Murray, 1998; Velmurugan et al., 1998; Velmurugan et al., 2000). Deletion analyses indicated that amino acid sequences near the carboxyl-terminal region of Rep1p harbor the nuclear targeting signal. In the absence of this signal, the protein was found to be dispersed throughout the cell, and could not support plasmid stability. Furthermore, when the deletion proteins were fused to an exogenous NLS (nuclear localization signal) derived from the SV40 T-antigen, their localization and potency in plasmid partitioning were restored to normal (Velmurugan et al., 1998). In our mutant analysis, it was important to establish that lack of plasmid stability, when observed, was not caused by the failure of the particular mutant protein to localize to the nucleus.

Mutant *REP1* genes were cloned into a yeast expression vector (pTS408; *CEN/URA3*) such that the N-terminus of each mutant was fused to C-terminus of GFP (Green Fluorescence Protein) and the expression of the chimera proteins was under the control of *GAL* promoter. The advantage of the hybrid protein is that it can be easily localized by fluorescence microscopy and at the same time tested for functionality. It has been established that the GFP-Rep1 fusion protein is active in

plasmid partitioning (Ahn et al., 1997). A [cir^0] host strain (FVY889-566) was used for localizing the GFP-Rep1p fusions and for assaying plasmid maintenance. The test plasmid for the stability assay harbored the *ADE2* and *LEU2* genes together with the 2 micron plasmid origin and the *STB* locus (Rep2p was also encoded by this plasmid and expressed from its native promoter). All experiments were carried out with above host strain transformed with the *ADE2/LEU2* reporter and a second *CEN* plasmid harboring a particular GFP-Rep1p variant. After inducing GFP-Rep1p mutant protein expression for about 6 hours at 30^{0}C, the yeast nucleus was stained with DAPI for another half an hour, and the live cells were subjected to fluorescence microscopy.

All the mutants used for functional analysis were checked, and were found to be localized in the nucleus. In Fig. 4.3, the localization of Rep1Y43A protein is shown as the representative pattern. The result is consistent with the previous finding that C-terminal of Rep1p is responsible for its nuclear targeting. Since the mutants were made within the first 330 amino acid of Rep1p, which contains 373 amino acids in total, the extreme C-terminus of the protein was intact in all of the mutants studied here.

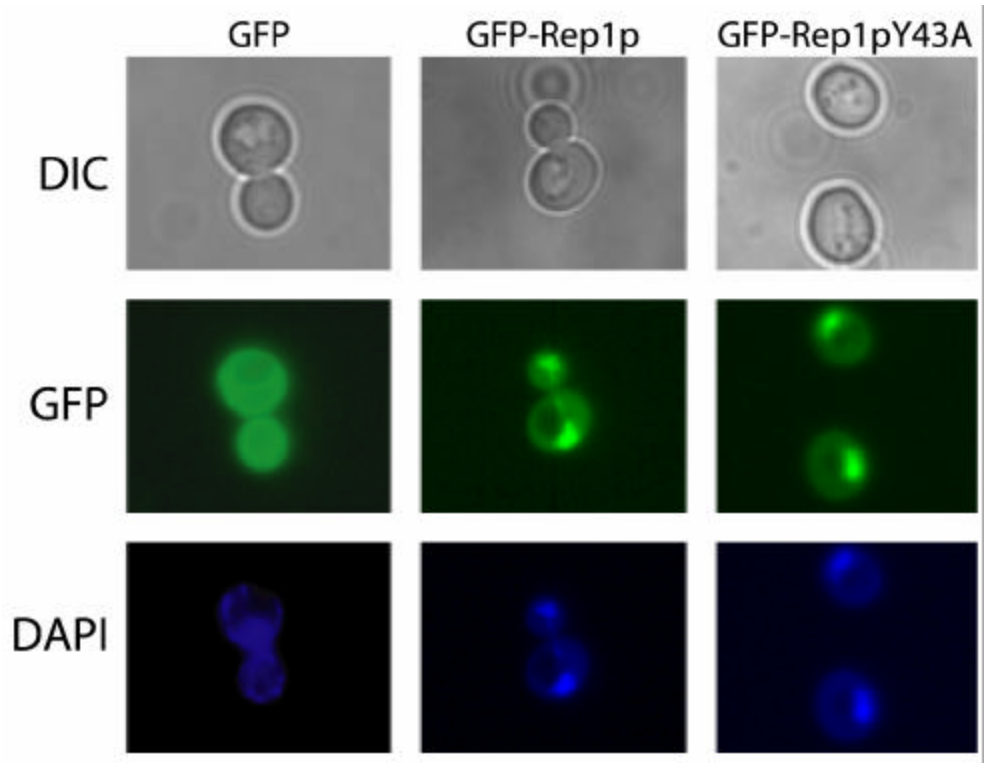


Figure 4.3 Localization of Rep1 mutant proteins. All mutants were found to localize to the yeast nucleus. Only the pattern for Rep1pY43A (third column) is shown here. The localization of GFP (first column) is shown as the negative control and that of GFP-Rep1p (second column) as the positive control.

4.3.3 Carboxyl-terminal regions of Rep1p and Rep2p in protein localization

As already pointed out, the amino acids at the C-terminal ends of Rep1p and Rep2p appear to function in plasmid stability by correctly targeting these proteins to the yeast nucleus (Velmurugan et al., 1998). Although not directly relevant to the main issues addressed in this chapter, I wished to know whether these amino acids by themselves could act as NLS sequences in yeast. The C-terminal 25 and 20 amino acids of Rep1p and Rep2p, respectively, were fused to the C-terminus of GFP in appropriate yeast expression plasmids (pTS408 derivatives), and the localization of the hybrid proteins was compared to that of GFP alone or GFP fused to the full-length Rep proteins (Fig. 4.4).

Consistent with the expectations from earlier experiments, the C-terminal peptides from both Rep1p and Rep2p localized GFP to the nucleus. Some signal from residual cytoplasmic GFP was also observed for both the Rep peptides. There was little or no cytoplasmic background fluorescence in the case of the GFP hybrids containing the entire Rep proteins. Note that, in theses experiments, the cells contained in addition to the GFP hybrids, the native form of the partner Rep protein as well: Rep1p in the case of the GFP-Rep2p or GFP-Rep2 peptide, for example. The patterns observed with the peptide fusions were similar to those seen earlier when a single Rep protein was expressed as a GFP-hybrid in a [cir^0] background (in the absence of its partner Rep protein). The sum of the old and the present data suggest that the efficient and highly localized nuclear organization

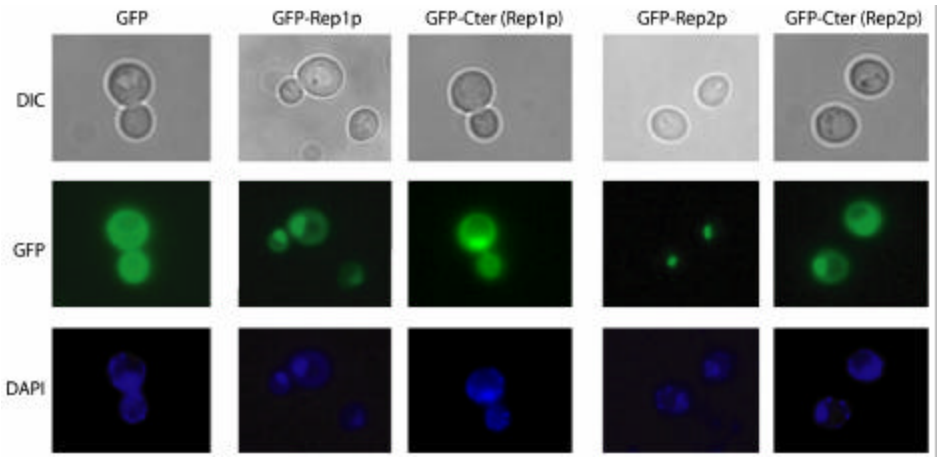


Figure 4.4 Localization of GFP hybrids containing the C-terminal peptides from Rep1p and Rep2p. GFP alone localizes throughout the yeast cell. The C-terminal peptides of Rep proteins direct GFP to the nucleus. The efficiency of nuclear targeting by the peptides is somewhat inferior to that of the full-length Rep proteins.

of the Rep proteins depends on the interaction between the two proteins even though the primary signal for nuclear transport resides at the C-terminus of each protein.

4.3.4 Interaction between Rep1 mutants and Rep2p

The yeast dihybrid assay was employed to test each of the Rep1p point mutants for interaction with Rep2p. To minimize potential false positives in the in vivo assay, two different assay systems were utilized. In the first system (the Philips James system, abbreviated here as PJ; James et al., 1996), there are three reporter genes under the control of the *UAS* sequence from the *GAL* promoter as chromosomal integrants: *HIS3* and *ADE2* and *LacZ*. The 'bait' in our experiments was the wild type Rep2p coding region fused to the sequences comprising the GAL4p DNA binding domain. The hybrid protein also contained the 15- amino acid S-tag peptide at the N-terminus (Kim and Raines, 1993). The 'prey' was constituted by the set of Rep1p mutants fused to the activation domain derived from Gal4p. Positive interaction between a certain Rep1p mutant and wild type Rep2p would be indicated by growth on medium lacking histidine (see Fig. 4.5). Lack of growth in the absence of histidine was taken as evidence for disruption of Rep1p-Rep2p interaction by a given mutation.

In cases where the assay revealed no interaction, it was important to ensure that the mutant Rep1p proteins were expressed normally. For this purpose,

yeast cell extracts were prepared, and total proteins were fractionated by electrophoresis in SDS-polyacrylamide gels. The gels were western-blotted, and probed with antibodies to Gal4p activation domain (Fig. 4.6). In every case tested, a band of approximately 60 kD, corresponding to the size of the Rep1p-activation domain fusion was detected.

To verify the interaction results, the Rep1p mutants were retested in the Roger Brent dihybrid system (abbreviated to RB; Finley and Brent, 1996). Here, wild type Rep2p was expressed as a chimera with the LexA repressor from the constitutive *ADH1* promoter. The Rep1p mutants were expressed as hybrids with an acid-rich transcription activation domain from the *GAL* promoter. The reporter cassette contained the *LEU2* gene whose transcription was controlled by three upstream copies of the LexA operator sequence placed as tandem repeats. Since the *REP1* chimeras are inducible in galactose, positive interaction between a Rep1p mutant and Rep2p would be declared by colony growth on plates lacking leucine in the presence of galactose but not dextrose (Fig. 4.7).

The interaction data for the mutant Rep1 proteins from the two dihybrid assay systems were in good agreement (Table 4.3). However, a few Rep1p mutants that showed weak or negative interaction with Rep2p in the PJ system were scored as positive interactors in the RB system. Note that both the bait and prey proteins in the PJ system were expressed from the *ADH1* promoter. In the RB system, the prey was under the control of the strong *GAL1* promoter, and the

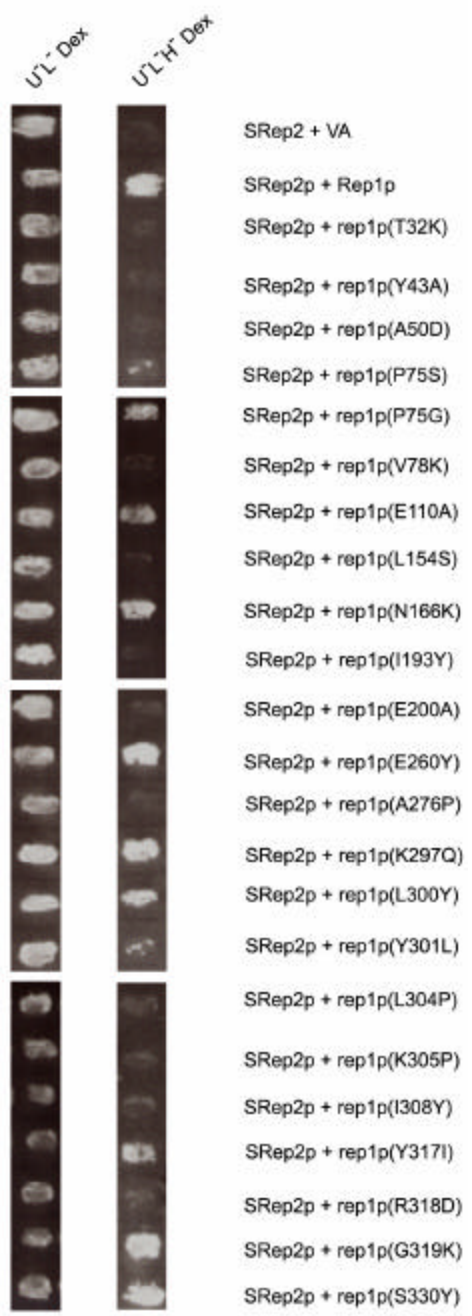


Figure 4.5 Interaction between wild type Rep2p and mutant Rep1 proteins in the PJ (Philip James) dihybrid assay. *HIS3* was used as the reporter gene. VA stands for vector containing the Gal4p activation domain alone (not fused to Rep1p or its point mutant derivatives). The protein listed left is the bait, and the protein listed right is the prey. The plasmids providing the bait and prey were maintained by keeping the selection for the *URA3* and *LEU2* markers harbored by them. U, L and H stand for uracil, leucine and histidine, respectively.

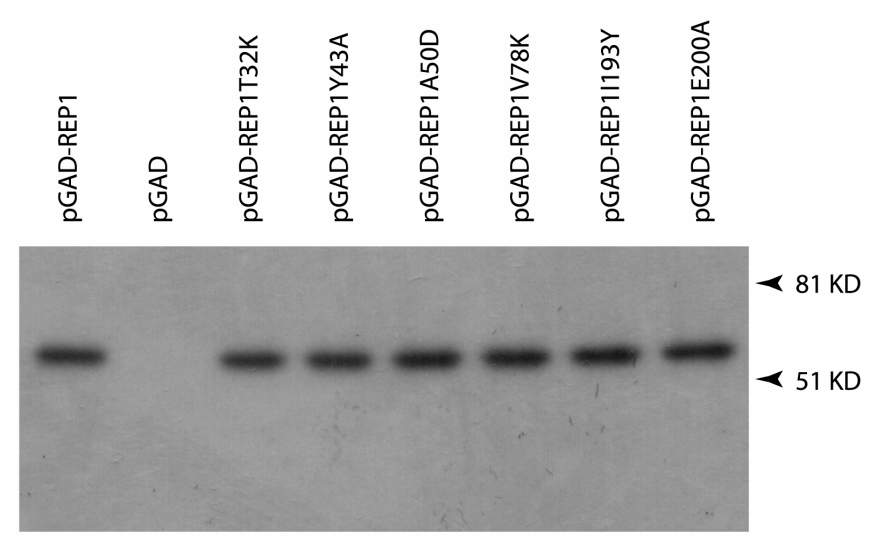


Figure 4.6 Expression of Rep1p mutants in the host strain used in the dihybrid assay. Whole cell proteins fractionated in an SDS-polyacrylamide gel by electrophoresis were probed using antibodies to Rep1p. The expression plasmids harboring the activation domain alone (pGAD) or wild type or mutant Rep1 proteins fused to the activation domain are indicated above the respective lanes. The Rep1p mutants shown here did not interact with Rep2p in the dihybrid assay.

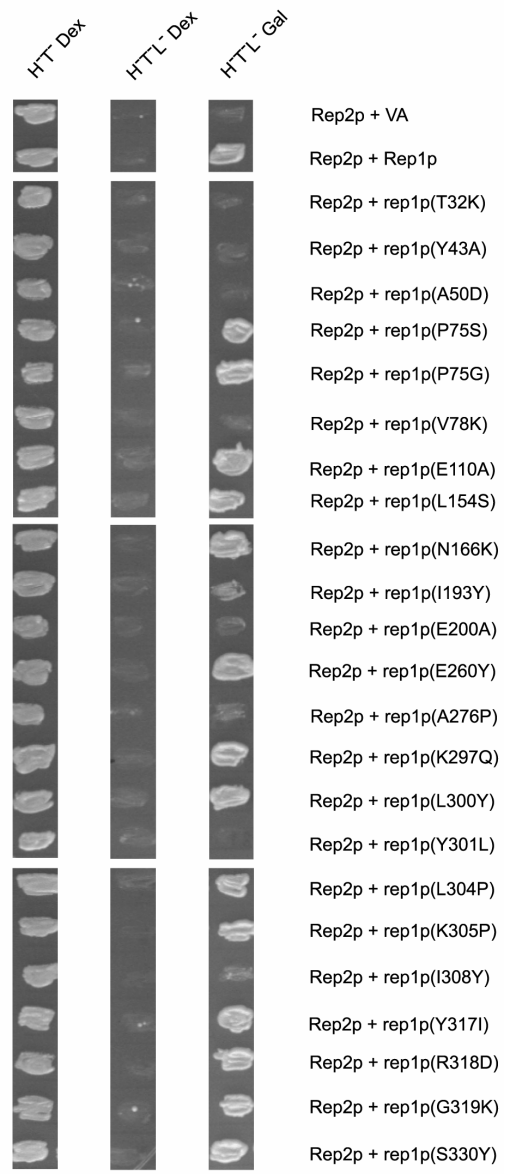


Figure 4.7 Interaction between wild type Rep2p and mutant Rep1 proteins in the RB (Roger Brent) dihybrid assay (Finley et al., 1996). *LEU2* was used as the reporter gene. VA stands for vector containing the transcriptional activation domain by itself (not fused to Rep1p or Rep1p mutants).

high protein levels might have permitted the detection of even weak interactions that were below the sensitivity level of the PJ system. However, this explanation does not account for two mutants Rep1p (Y301L) and Rep1p (I308Y), both of which were weak interactors in the PJ system and tested negative in the RB system. Nevertheless, as was revealed by further functional assays, the negative and the weak interactors were unable to support normal plasmid stability (see below).

In general, Rep1p mutations that affect Rep2p interaction are not strongly clustered to a specific region of the protein primary structure (Table 4.3; Fig. 4.9). However, we note that four mutations (T32K, Y43A, A50D and V78K) within the N-terminal 100 amino acids abolish its interaction with Rep2p. This result is consistent with previous deletion analyses (Sengupta et al., 2001; Velmurugan et al., 1998) demonstrating that the N-terminal portion of Rep1p is required for its interaction with Rep2p. We further note that several mutations near the C-terminal portion of Rep1p (A276P, Y301L and L308Y) and a few mutations near the midsection of the protein also resulted in loss or diminution of interaction with Rep2p. Thus, it seems likely that there may be more than one contact face between Rep1p and Rep2p, or the interaction surface on Rep1p may be formed by the congregation of amino acids from different regions of the protein.

4.3.5 Interaction between Rep1p mutants and *STB*

Results from previous monohybrid assays showed that Rep1p and Rep2p can interact with *STB*, this interaction being independent of each other (S. Velmurugan and M. Jayaram, unpublished data). In vitro gel shift studies have suggested that one or more host factors (supplied as a urea-solubilized and dialyzed extract from a [cir^0] yeast strain) might be required to mediate binding between a Rep protein and *STB* (Hadfield et al., 1995). In a more recent study using the southwestern assay, the C-terminal portion of Rep2p has been shown to possess non-specific DNA binding activity (Sengupta et al., 2001). Assuming that *STB*-Rep interaction is central to plasmid segregation, we wished to know whether some of the Rep1p mutants are defective in associating with *STB*, and whether such mutants would be uniformly defective in effecting plasmid partitioning.

The interaction between the Rep1p mutants fused to the Gal4p activation domain and *STB* was tested using a *HIS3* reporter driven by its minimal promoter (weak expression). The *STB* sequence (approximately 375 bp consisting of five copies of the 65 bp consensus units) was placed around 120 bp upstream of the *HIS3* transcription start site. This construct, in which *STB* serves as the *UAS* for *HIS3* transcription, was chromosomally integrated in the monohybrid tester strain YM4271. The weak constitutive transcription can produce only nominal levels of the His3 protein. As a result, the strain grows extremely poorly or not at all under

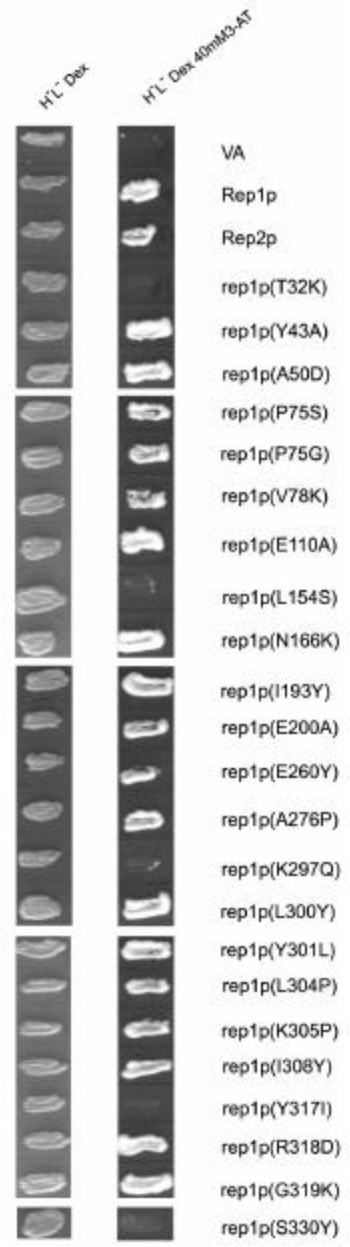


Figure 4.8 Interaction between Rep1p mutants and the *STB* element in monohybrid assay. The second column shows the growth of the strain in the presence of 40 mM 3-AT. VA stands for vector containing the Gal4p activation domain alone (not fused to Rep1p or its point mutant derivatives).

challenge by the His3p-specific inhibitor 3-AT (3-aminotriazole) in the 15 to 40 mM range. Under our experimental conditions, there was no growth at all above 20 mM 3-AT. If a protein carrying an activation domain can bind to *STB*, enhanced transcription of *HIS3* and consequently resistance to 3-AT will ensue. This provides the basis for determining whether a given Rep1p mutant is capable of interaction with *STB* or not. The Rep1p mutants fused to the activation domain were introduced into the host strain as part of a *LEU2* containing plasmid ($pGAD_{424}$), and were expressed from the *ADH1* promoter.

The results of the monohybrid assays are displayed in Fig. 4.8 and summarized in Table 4.3. Of the 23 Rep1p mutants, five were found to be defective in *STB* interaction. Of these three were in the C-terminal part (K297Q, Y317I and S330Y), and one was in the N-terminal region (T32K). Interestingly, the latter one mutant was also negative in Rep2p interaction.

4.3.6 Functional analysis of Rep1p mutants by plasmid stability assay

Since our collection revealed mutations in the Rep1 protein that disrupt Rep2p interaction or *STB* interaction or both, the next step was to examine the behavior of these mutants in plasmid segregation. The reporter plasmid (cp22) for the stability assay contained the *STB* element, the *REP2* locus and the *ADE2* gene as a color marker. As indicated previously, all assays were carried out in a [cir^0] strain using the GFP-Rep1p fusion proteins expressed from a *CEN* plasmid by the

GAL10 promoter (pTS408). Recall that these GFP hybrids were the ones used for testing the nuclear localization of the Rep1p variants. As a result any spurious results due to expression problems or mislocalization could be ruled out. Transformants containing the reporter plasmid and the expression plasmids for GFP-Rep1p (or GFP-Rep1p mutants) were maintained on galactose selective plates to retain both plasmids and to induce Rep1p or mutant Rep1p expression. Individual colonies were picked, and after appropriate dilution, cells were plated out on YP-galactose plates. Thus, induction was continued while the selection for the reporter plasmid was removed. The *CEN*-plasmids expressing Rep1p or its variants was stably maintained (better than 95% of cells were plasmid positive) under these conditions. Red and white colonies, indicating loss or retention, were counted to estimate the stability index (SI) as the number of white colonies divided by the sum of white and red colonies. Colonies with red sectors were counted as white if the sector size added up to less than one fourth the colony size and as red if the sectors constituted greater than one fourth the colony size (as estimated roughly by visual observation). The stability results are summarized in Table 4.3 and Fig. 4.9. For wild type Rep1p (functioning as the GFP hybrid), the SI was approximately 62 (Velmurugan et al., 1998).

Table 4.3 The functional consequences of Rep1p point mutations that disrupt interactions with Rep2p and/or the *STB* element

Rep1p point mutants	Interaction between Rep1 mutant proteins and Rep2p (P.J. system)	Interaction between Rep1 mutant proteins and Rep2p (R.B. system)	Interaction between Rep1 mutant proteins and *STB*	Stability Index
T32K	-	-	-	< 5
Y43A	-	-	+	< 5
A50D	-	-	+	< 5
P75S	weak	+	+	54
P75G	+	+	+	63
V78K	-	-	+	< 5
E110A	+	+	+	61
L154S	-	+	-	< 5
N166K	+	+	+	63
I193Y	-	-	+	< 5
E200A	-	-	+	< 5
E260Y	+	+	+	62
A276P	-	-	+	< 5
K297Q	+	+	-	< 5
L300Y	+	+	+	59
Y301L	weak	-	+	< 5
L304P	Weak	**+**	**+**	**< 5**
K305P	Weak	**+**	**+**	**< 5**
I308Y	weak	-	+	< 5
Y317I	+	+	-	< 5
R318D	Weak	**+**	**+**	**< 5**
G319K	**+**	**+**	**+**	**< 5**
S330Y	+	+	-	< 5

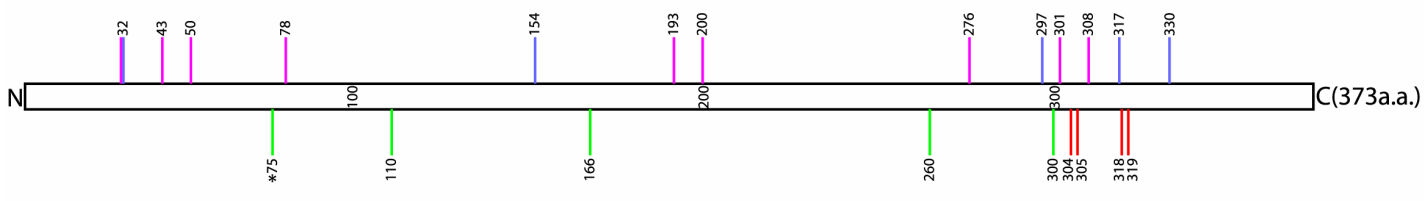


Figure 4.9 Summary of the experimental results characterizing the Rep1p mutants. Each mutant is indicated by a colored bar and the number of the amino acid position altered by the mutation. The specific nature of the substitutions is listed in Table 4.2. The violet bars (—) indicate Rep1p mutants that interact with Rep2p but not *STB*. The blue bars (—) stand for mutants that interact with *STB* but not Rep2p. The one mutant in which both Rep2p and *STB* interactions have been eliminated is shown by a doublet of violet and blue bars. All of the above mutants are unable to support plasmid partitioning in a standard stability assay. The Rep1p variants denoted by the green bars (—) are normal in their interactions with Rep2p and *STB*, and are functional in plasmid partitioning. The mutants represented by the red bars (—) can not support plasmid segregation, even though they interact both with Rep2p and with *STB*. The asterisk indicates that that two mutants tested at position 75 are identical in phenotype. The interaction results with Rep2p presented here are those derived from the Roger Brent (RB) dihybrid assay system.

4.3.7 Are Rep1p mutants dominant negative over wild type Rep1p?

In the general model for plasmid segregation, assembly of an active partitioning complex is predicated upon Rep1p-Rep2p interaction as well as the Rep protein interactions with *STB*. The results assembled in Table 4.3 are consistent with this model. A reasonable prediction from this model is that a mutant Rep1p (incompetent in either Rep2p interaction or *STB* interaction), when present in molar excess over its wild type counterpart, will poison the complex. Plasmid stability should decrease as a result.

To test the predicted dominant negative phenotype of Rep1p mutants, we chose one mutant that can not interact with Rep2p (Rep1pV43A) and one that can not interact with *STB* (Rep1pK297Q). In the stability assay, the mutant proteins were expressed from the *GAL10* promoter, while the wild type Rep1 protein was expressed from its native promoter. Thus, one would expect to observe normal wild type function in dextrose grown cells (the mutant protein being repressed), whereas this function should be interfered with in galactose grown cells. Two 2 micron plasmid derivatives, each containing the *ADE2* marker were employed as the reporters. The *ADE2* gene was placed within a non-coding region between *STB* and the *RAF1* gene in one of the reporters, and it was inserted in the *FLP* gene in the other. The Flp protein does not play a detectable role in plasmid partitioning, as determined by standard genetic assays. Both plasmids exhibit high stability in dextrose and galactose in the presence of the native plasmid

partitioning system (Rep1p, Rep2p and *STB*) (Table 4.4). Rather unexpectedly, similar stability levels were obtained in dextrose and galactose even when the expression vectors containing the mutant Rep1 proteins were also resident in the cells (Table 4.4). There are two reasons for the higher stability values in Table 4.4 than those in Table 4.3. First, the assays were carried with wild type or mutant Rep1p proteins that were not fused to GFP. The GFP fusion proteins are slightly less active than the corresponding native proteins. Second, the two reporter plasmids had different configurations than the one used for the assays depicted in Table 4.3, and these had intrinsic higher stabilities.

Because of the counter intuitive nature of the above results, we worried whether the mutant proteins were really overexpressed from the *GAL* promoter under our experimental conditions. To clarify this point, western blot analysis was performed on fractionated whole cell proteins using antibodies to Rep1p (Fig. 4.10). Although the antibodies cannot distinguish between the wild type and mutant forms of the Rep1 proteins, the increased steady state levels of Rep1p plus mutant Rep1p in galactose grown cells relative to dextrose grown cells would indicate that mutant expression was inducible. The protein levels under dextrose growth provide a base line for Rep1p expression from its native promoter.

As is clear from the combined results from Table 4.4 and Fig. 4.10, efficient plasmid segregation can be mediated when the cell contains a mixture of

Table 4.4 Rep1p mutants are not dominant negative over wild type Rep1p

Test plasmid	Expression vector	SI on YPD (%)	SI on YPGal (%)
2micron-ADE2	———	99.3 ± 0.6	99.8 ± 0.3
	pBM272	100.0 ± 0.0	100.0 ± 0.0
	pBM272-REP1Y43A	99.4 ± 0.8	99.4 ± 0.6
	pBM272-REP1K297Q	98.9 ± 0.9	99.3 ± 1.0
2micron-ADE2(flp)	———	87.1 ± 9.5	91.5 ± 4.4
	pBM272	83.3 ± 7.4	88.9 ± 5.5
	pBM272-REP1Y43A	84.4 ± 8.1	89.2 ± 8.0
	pBM272-REP1K297Q	85.2 ± 6.6	79.2 ± 7.6

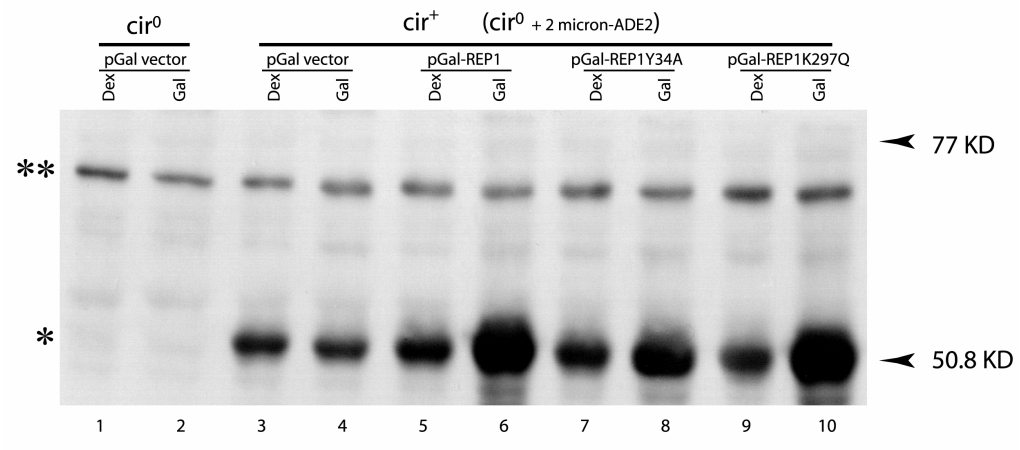


Figure 4.10 Western blot assay for the overexpression of Rep1p mutants. Lane 1 and 2 were loaded with [cir^0] cell extracts, and served here as negative controls. Anti-Rep1p antibody was used for the western analysis, so that both wild type and mutant forms of Rep1p were detected by the antibody (*). A non-specific band, indicated by **, appeared in all samples, and provided a control for equal loading of total proteins.

wild type and mutant Rep1 proteins, even with the mutant being present in much higher abundance.

4.4 Discussion

4.4.1 Phenotypes of the Rep1p mutants support a partitioning mechanism requiring Rep1p-Rep2p-*STB* interactions

The Rep1 mutants characterized in this study could be divided into five groups (Fig. 4.9). Group 1 (8 mutants) failed to interact with Rep2p but was normal in *STB* interaction whereas group 2 showed the reverse phenotype of being able to interact with Rep2p but not *STB*. One mutant showed no interaction with Rep2p or with *STB*, and was classified under group 3 in anticipation of identifying more such mutants in the future. All members of groups 1-3 were non-functional in plasmid partitioning. This is consistent with the proposed model that requires interactions of Rep1p with both Rep2p and *STB* for effective plasmid partitioning. Six Rep1p variants, included in group 4, behaved as wild type Rep1p in Rep2p interaction, *STB* interaction and support of plasmid stability. The most interesting class was the four members of class 5, each of which was normal in Rep2p and *STB* interactions but turned out to be non-functional in plasmid partitioning. These could be defective in Rep1p-Rep1p interactions (not tested here) or interaction with a host factor or in some other step of plasmid segregation

that is epistatic to Rep1p-Rep2p-*STB* association. One of these variants (Rep1pL304P) was found to be defective in interaction with the yeast condensin subunit Brn1p with which wild type Rep1p interacts in a dihybrid assay (see Chapter 5, section 5.2.2). The other three were normal in their interaction with Brn1p. As described in Chapter 5, we have obtained strong circumstantial evidence that the yeast cohesin complex is important in plasmid partitioning and acts via the Rep-*STB* system (Mehta et al., 2002). Because of the mutually cooperative roles of cohesin and condensin complexes in chromosome partitioning, it is quite possible that the partitioning defect exhibited by Rep1pL304P may result from its loss of interaction with the yeast condensin complex. However, further work is required for testing this hypothesis critically.

4.4.2 Unanswered questions to be addressed in the future

1. Can Rep1p mutants that can not self-interact be identified in our mutant library? If so, will these mutants fail to mediate normal plasmid partitioning?

2. Is the interaction between Rep1p and Brn1p revealed by the dihybrid test functionally relevant? Can interaction be established with other components of the yeast condensin complex (Smc2p, Smc4p, Ycs4p and Ycs5p) as well? Can one monitor changes in the organization of the plasmid cluster or the separation of the duplicated plasmid clusters when one or more subunits of the condensin complex are inactivated by conditional mutations?

3. Is the non-dominance of the overexpressed inactive Rep1p mutant under normal expression of wild type Rep1p really due to the exclusion of the mutant protein from the partitioning complex? We should be able to answer this question by doing chromatin immunoprecipitation with antibodies to tagged versions of wild type and mutant Rep1 proteins and following their association (or lack thereof) with *STB*.

CHAPTER 5

Host Factors Involved in 2 Micron Plasmid Partitioning

5.1 Abstract

1. A dihybrid screen has revealed host factors that interact with the 2 micron stability system. One of these is a subunit of the yeast condensin complex (Brn1p), while a second one (Fun30p) belongs to the *SNF2* class of transcriptional regulators involved in chromosome remodeling.

2. We have not been able to establish a functional role for Brn1p or Fun30p in the maintenance of the 2 micron plasmid. However, a Rep1p mutant, carrying a point mutation (L304P) does not interact with Brn1p and cannot support plasmid stability. Because of the non-specific association of yeast condensin complex to DNA, we have focused on the related cohesin complex for its possible role in plasmid partitioning.

3. *In vivo* monohybrid and dihybrid assays have been employed to confirm the specific association of the cohesin complex with the *STB* locus inferred from chromatin immunoprecipitation and to establish the role of the Rep proteins in this process.

4. The integrity of the mitotic spindle is essential for the cohesin subunit Mcd1p to associate with *STB*. Spindle disassembly has no effect on the binding of

Mcd1p to a chromosomal cohesin binding site. When the spindle is allowed to reform after depolymerization with nocodazole followed by removal of the drug, cohesin becomes reassociated with *STB* with the same kinetics as the kinetics of spindle restoration.

5.2 Background

As a benign molecular parasite, the 2 micron plasmid utilizes the host DNA replication and transcription machineries for its own duplication and gene expression. Moreover, recent studies suggest that this channeling of important cellular components may extend to the process of plasmid partitioning as well (Mehta et al., 2002). Comparison of the dynamics of a fluorescently tagged 2 micron plasmid to a similarly tagged chromosome or a centromeric plasmid shows that the segregation kinetics of all three are quite similar during the yeast cell cycle (Chapter 3; Velmurugan et al., 2000). This is not necessarily so for a tagged *ARS* plasmid. In addition, in strains harboring mutations that affect distinct steps of the chromosome segregation pathway (namely, mutations in *IPL1*, *CTF7*, *CTF13*, *CTF14*/*NDC10* and *NDC80*), the chromosomes and the 2 micron plasmid almost always missegregate in tandem (Chapter 3; Mehta et al., 2002; Velmurugan et al., 2000; Wong et al., 2002). Furthermore, where tested, this strong correlation is dependent on the Rep proteins. The absence of the *STB* locus *in cis* (as in the case of an *ARS* plasmid) or the lack of one or both of the Rep proteins (as in a [cir^0] strain or its engineered derivatives) breaks this correlation, and plasmids segregate to opposite cell poles independent of chromosomes. Even more strikingly, chromatin immunoprecipitation assays have revealed that subunits of the yeast cohesin complex (Mcd1p, Smc1p, Smc3p) associate with *STB* element, likely recruited by the Rep proteins (Mehta et al., 2002).

The emerging picture suggests that the organization of the plasmid molecules and their spatial location may be directly relevant to partitioning. A high order molecular complex that includes plasmids tightly associated with the Rep proteins (presumably host proteins are also part of this complex) is the likely basis for the clustering observed when tagged plasmids are followed by fluorescence microscopy. Within a yeast nucleus, a plasmid cluster shows a high propensity to be close to the mitotic spindle, in particular the spindle pole (Velmurugan et al., 2000). It is the cluster that forms the segregating unit, and little or no declustering has been observed during segregation. It is not unreasonable to think of the aggregate of 60 or so copies of the plasmid as the equivalent of a small yeast chromosome (we call it a 'plasmasome') whose segregation pathway overlaps with that of the chromosomes or is coordinately regulated with the latter. If this hypothesis is correct, the Rep-*STB* system is the likely to mediate the coupling between plasmid and chromosome segregation.

5.3 Results

5.3.1 Screening the yeast genome for host factors that interact with the Rep proteins

To test the idea that the 2 micron partitioning system functionally interacts with host factors, a genome wide two hybrid assay was carried out using the Rep1

and Rep2 proteins as the baits. Details of the methodology can be seen under 'Materials and Methods' (Chapter 2). A list of the genes that tested positive in this screen is given under Table 5.1.

The positive interactors identified with the Rep1p bait included portions of the predicted polypeptide products from the following genes: *BRN1* (amino acids 332-648), *SET1* (amino acids 243-1080) and *SAP185* (amino acids 863-1058). In addition, a partial clone of the *FUN30* gene (amino acids 187-1131) and the *YPR169w ORF* (encoding amino acids 268-514) were obtained in this screen. The Rep2p bait also fished out the same *BRN1* clone (amino acids 332-648) as did Rep1p. The other positives with Rep2p were a portion of the *YBR095C ORF* (amino acids 342-453) and a potential coding sequence derived from the Ty1 transposon of yeast, although the latter interaction was quite weak. When retested by the dihybrid method, the majority of the clones, with the exception of *SAP185* and *YRP169w*, turned out to interact with both Rep1p and Rep2p. This might either represent their true interaction with each of the Rep proteins or might simply reflect the fact that Rep1p and Rep2p interact with each other. This issue is addressed later. Note that the dihybrid assays were done in a [cir^+] host strain, and hence Rep1p and Rep2p were endogenously supplied by the native 2 micron circle.

BRN1 is essential for yeast viability, and a temperature sensitive mutation in this gene leads to partially elongated chromosome masses (indicative of

Table 5.1 Results of a dihybrid screen for yeast proteins that interact with Rep1p or Rep2p

Screening bait	Locus	Interaction with Rep1p	Interaction with Rep2p	Function
Rep1p	/	+	weak	/
	BRN1 (332 a.a.-648 a.a.)	+	+	Condensin subunit, involved in chr. segregation
	SET1 (243 a.a.-1080 a.a.*)	+	weak	Histone-lysine N-methyltransferase histone methylation
	SAP185 (863 a.a.-1058 a.a.*)	+	_	Protein ser/thr phosphatase mitotic G1/S transition
	FUN30 (187 a.a.-1131 a.a.*)	+	+	Shows homology to *SNF2*
	YPR169w (268 a.a.-514 a.a.*)	+	_	/
Rep2p	/	+	+	/
	BRN1 (332 a.a.-648 a.a.)	+	+	Condensin subunit, involved in chr. segregation
	/	+	weak	/
	inside Ty1	weak	weak	/
	BRN1 (332 a.a.-648 a.a.)	+	+	Condensin subunit, involved in chr. segregation
	/	+	+	/
	YBR095c (342 a.a.-453 a.a.*)	+	+	/

* The asterisk on the amino acid number denotes that the particular amino acid is the very C-terminal residue of that protein

defective chromosome condensation) and defective sister chromatid segregation under the restrictive condition (Lavoie et al., 2000; Ouspenski et al., 2000). This phenotype resembles the anaphase defect observed for barren mutants in *Drosophila*, the *smc2* mutants in *Saccharomyces cerevisiae*, *cut3* and *cut14* mutants in *Schizosaccharomyces pombe*, and *mix1* mutants in *Caenorhabdites elegans* (Bhat et al., 1996; Hirano et al., 1997; Saka et al., 1994; Sutani et al., 1999; Uhlmann, 2001). All of these genes encode homologues of a component of *Xenopus* condensin, a complex that has been demonstrated to mediate chromosome condensation *in vitro*. From the cell cycle progression patterns observed in *S. cerevisiae brn1* mutants, it has been inferred that Brn1p performs an essential function between early S phase and early M phase, the period of the cell cycle during which condensation is established and maintained (Lavoie et al., 2000; Ouspenski et al., 2000).

The *FUN30* gene has been previously recognized as important for chromosome integrity and segregation in *Saccharomyces cerevisiae* (Ouspenski et al., 1999), even though it is not essential for cell viability. When overexpressed, it causes defective partitioning of a reporter minichromosome. Fun30p shows homology to the *SNF2* type of transcriptional regulators that are associated with chromatin remodeling complexes (Sudarsanam and Winston, 2000).

To confirm the results of the initial dihybrid analysis, full length *FUN30* and *BRN1* genes were cloned and were retested by the dihybrid assay in [cir^+] or

[cir^0] backgrounds. The results for Brn1p-Rep proteins interaction are shown in Fig. 5.1 and discussed below.

5.3.2 Preliminary analysis with *BRN1* and *FUN30*

Plasmid stability in *brn1* mutants:

The secondary dihybrid test verified the interaction of the full-length Brn1 protein with Rep1p and Rep2p. Among the collection of the 'loss of function' Rep1p mutants (Chapter 4), we identified one, Rep1pL304P, which failed to interact with Brn1p. Since Rep1pL304P interaction with Rep2p and *STB* DNA are normal (from previous analysis; see Chapter 4, Table 4.3), its inability to support plasmid stability may result from the loss of its interaction with Brn1p.

Positive interaction of full-length Brn1p with either Rep1p or Rep2p was also observed in a [cir^0] background, indicating that the interaction with one Rep protein was independent of the other. We also tested the interaction between Brn1p and the *STB* element in yeast monohybrid system (Fig. 5.2). Brn1p was found to interact with *STB* in a [cir$^+$] background but not in a [cir^0] background. Therefore, this interaction must be indirect, and is likely mediated through the Rep proteins (and perhaps even some host proteins).

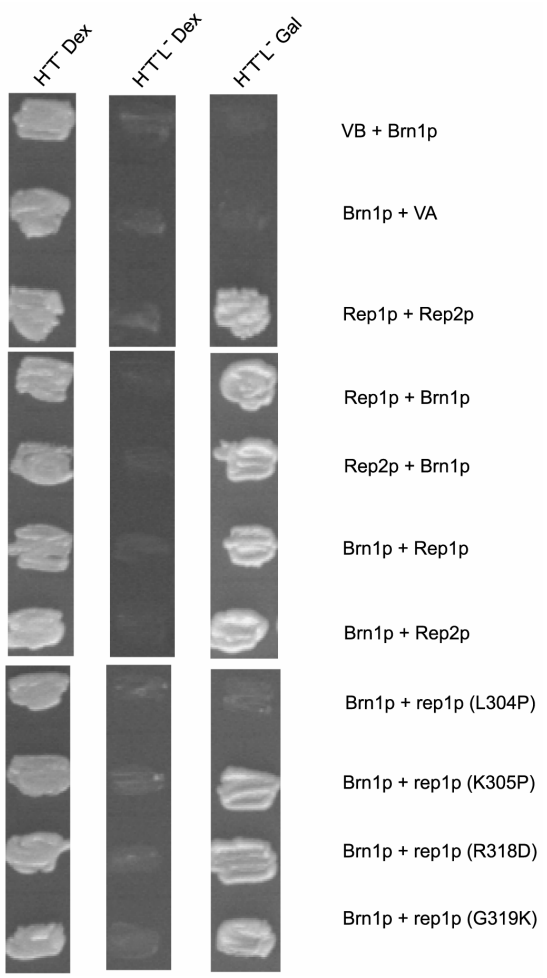


Figure 5.1 Interaction between Brn1p and Rep proteins in the RB (Roger Brent) dihybrid assay (Finley et. al., 1996). *LEU2* was used as the reporter gene. VA stands for vector containing the transcriptional activation domain by itself (not fused to Rep1p or Rep1p mutants). For a given binary protein combination, the protein listed before the plus sign was the bait (fused to LexA), and the protein following the plus sign was the prey (fused to the activation domain).

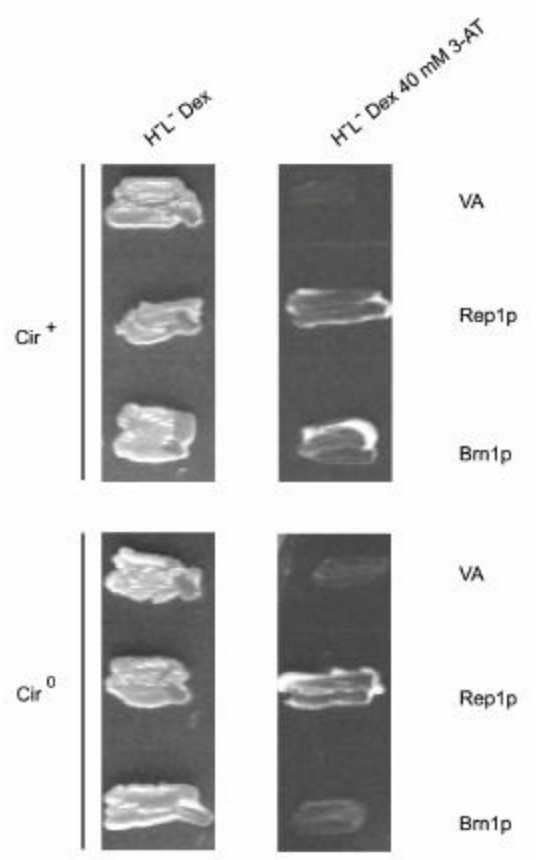


Figure 5.2 Interaction between Brn1p and the *STB* element in yeast monohybrid assay. The second column shows the growth of the strain in the presence of 40 mM 3-AT. VA stands for vector containing the Gal4p activation domain alone (not fused to Rep1p or its point mutant derivatives). Strain background ([cir^+] or [cir^0]) is indicated at the left of the column.

We wished to check whether *BRN1* had any effect on the stability of the 2 micron plasmid. Three *Ts* mutant strains provided by Dr. I. Ouspenski (then at the Baylor College of Medicine and now at NIH) were used for this purpose. Because of the essential nature of the *BRN1* gene, the stability assays were carried out at 34ºC that permitted growth although with a longer generation time than at 30ºC. Two of the strains (*brn1-20* and *brn1-34*) showed a high rate of plasmid loss that was comparable to that observed in [cir^0] strains. Curiously, the third strain (*brn1-60*) displayed almost normal plasmid stability. This unexpected result prompted us to test the status of the presence or absence of native 2 micron plasmid in these strains by colony PCR. We found to our surprise that the former two strains had lost the plasmid, whereas the latter still contained it.

To eliminate the possibility that potential allelic effects might have been responsible for the differences in plasmid stability, we did the stability test again after reintroducing the native 2 micron circle into the *brn1-34* strain by crossing them with an isogenic wild type [cir^+] strain of the opposite mating type. After sporulation of the diploids and tetrad dissection, the temperature sensitive haploids (containing the *brn1* mutation) were screened by colony PCR to verify their [cir^+] status. The stability of the reporter plasmid was restored to more or less normal in the [cir^+] derivatives of the *brn1-34* strain. These results cannot eliminate nor substantiate a role for *BRN1* in the maintenance of the 2 micron plasmid. If *BRN1* is required for plasmid partitioning, the effect is too subtle to be

revealed by the semi-permissive temperature employed in the stability assays. The *brn1-20* strain was also crossed with a wild type [cir^+] strain of the opposite mating type, but difficulties in sporulation prevented the stability test being conducted as described above for *brn1-34*.

At this time, the potential involvement of Brn1p in plasmid segregation is only speculative, and needs to be investigated more carefully. Preliminary attempts in this direction were not encouraging because of the non-specific association of Brn1p with plasmids in chromatin immunoprecipitation assays, even in the absence of an intact partitioning system. This lack of strict specificity has been observed by others as well in *in vitro* assays with condensin complexes from different organisms (Kimura and Hirano, 2000). By contrast, the cohesin complex (which is also central to chromosome segregation) shows a high degree of discrimination between its authentic target sequences and non-specific DNA (Laloraya et al., 2000; Megee et al., 1999). As a result our initial focus has been redirected from the condensin complex to the cohesin complex (see below).

Plasmid stability in the *Fun30D* background:

As described for *BRN1*, the interaction between full-length Fun30p and Rep proteins were tested in the same dihybrid system. While Fun30p could interact with either Rep1p or Rep2p in the [cir^+] strain, its interaction with Rep2p (but not Rep1p) was abolished in the [cir^0] strain. Therefore, we conclude that the

association between Fun30p and Rep2p is indirect, and is mediated via Rep1p (data not shown).

In the monohybrid test, Fun30p showed interaction with *STB* in the [cir^+] but not the [cir^0] genetic background (data not shown). This observation is consistent with the dihybrid result that Fun30p interacts with Rep1p and through the latter associates with Rep2p as well. We conclude that the binding of Fun30p to *STB* must be mediated by the Rep proteins.

Since *fun30* deletion strains are viable (unlike the *BRN1* situation) with no apparent growth problems, we could readily monitor plasmid maintenance defects in such strains. A *fun30* deletion strain and its wild type parent strain were obtained from Invitrogen Corporation (Carlsbad, California), and plasmid stability was assayed in the two. Again, we encountered a situation curiously similar to that described for the *brn1* mutants. The deletion strain showed a high rate of plasmid loss relative to the wild type. Colony PCR showed that the former had lost the native 2 micron plasmid despite being derived from a [cir^+] parent. Next, we disrupted *FUN30* by the targeted insertion of *URA3* in a [cir^+] wild type strain (CRY1), and measured plasmid stability after verifying the [cir^+] status of the disruptant. In this background, plasmid stability was restored to near normal values. We conclude that the role of *FUN30* in plasmid maintenance is not critical.

Summary of the *BRN1* and *FUN30* data

The results from the analyses of *BRN1* and *FUN30* so far are tantalizing but inconclusive. The fact that [cir^0] cells arise spontaneously in the population when the *BRN1* gene is mutated or the *FUN30* gene is deleted, are consistent with their role in plasmid maintenance in yeast. However, *BRN1* being essential for chromosome segregation, it would be necessary to look for *brn1* mutations that affect plasmid stability in a measurable way with no effects or only minimal effects on chromosome segregation. If plasmid and chromosome segregation pathways are coupled (several pieces of circumstantial evidence suggest this to be the case), it is unrealistic to expect that such mutations can be recovered. Similarly, it may be possible to identify in a *fun30* deletion background mutations in a second gene that might give clear cut plasmid instability (synthetic effect). Once again, because of plausible effects on chromosome segregation, it is unclear whether such mutations can be revealed. Because of these uncertainties, we have decided to postpone further work on *BRN1* and *FUN30*. Instead, we have focused on the potential role of the yeast cohesin complex in the partitioning of the 2 micron plasmid (see below).

5.3.3 Cohesion subunit Mcd1/Scc1 protein associates specifically with *STB* element

The yeast cohesin complex plays a central role in chromosome segregation by establishing sister chromatid pairing during the S phase and maintaining it until chromosomes are ready to be separated during anaphase. Due to the similar segregation kinetics of 2 micron plasmid and the chromosomes during the yeast cell cycle, we considered the possibility that the plasmid might utilize the cohesin complex to ensure its stable partitioning. Part of the rationale for making this hypothesis was based upon the finding that a condensin subunit (Brn1p), and perhaps the condensin complex, interacts with the 2 micron plasmid partitioning system. Both cohesin and condensin contain structurally conserved components (the SMC proteins), and play distinct but mutually cooperative roles during chromosome segregation. We chose to investigate cohesin first because of its greater specificity over condensin in DNA binding.

Other workers in our laboratory showed by chromatin immunoprecipitation (ChIP) that a central component of the yeast cohesin complex, Mcd1p, can associate with the *STB* element with a high degree of specificity (Mehta et al., 2002). In brief, chromosomal DNA, sheared to an average length of 500 bp, was immunoprecipitated using Mcd1p directed antibodies and probed for the presence of 2 micron DNA by PCR. The *STB* DNA was present in immunoprecipitates from a [cir^+] strain or a [cir^0] strain expressing

Rep1p and Rep2p and harboring an *STB*-containing reporter plasmid. Other regions of the 2 micron plasmid spanning the replication origin or internal to the *REP1*, *REP2* and *FLP* genes were not precipitated by the Mcd1p antibody. Further ChIP experiments showed that Mcd1p-*STB* association was dependent upon the simultaneous presence of Rep1p and Rep2p. Other components of the cohesin complex such as Smc1p and Smc3p also showed interaction with *STB* as judged by ChIP assays. Finally, when one of the cohesin subunits carried a *Ts* mutation (say, Smc3p), a second subunit (say, Mcd1p) failed to show *STB* association at the non-permissive temperature but showed normal association at the permissive temperature. Based on the sum of the ChIP results, we concluded that it is the whole cohesin complex (perhaps in its preassembled form) that interacts with the 2 micron plasmid partitioning system.

Given the critical role of cohesin in the faithful distribution of sister chromatids to daughter cells during cell division, together with the apparent coupling between the segregation of the 2 micron plamsid and that of the chromosomes, it would seem reasonable to suppose that the cohesin complex is invovled in plasmid partitioning. The fact that cohesin association is highly specific to the components of the plasmid stability system strengthens this proposition. An important question is whether the protein-protein and DNA-protein interactions indicated by the ChIP results can be verified by independent

in vivo assays. As described below, I have employed the dihybrid and monohybrid assays to answer this question.

5.3.4 Requirement of the Rep proteins for Mcd1p-*STB* interaction is corroborated by *in vivo* dihybrid and monohybrid assays

In the first set of dihybrid assays used to probe the potential interaction of Mcd1p with the Rep proteins, the former was fused to the Gal4p activation domain (AD) and the latter were fused to Gal4p DNA binding domain (BD). The Gal4p target site served as the *UAS* for the *HIS3* reporter gene. In this assay, Mcd1p interacted with both Rep1p and Rep2p, as indicated by growth of the strain on His minus plates (Fig. 5.3). Subsequently, the experiment was repeated by reversing the DNA binding and activation domain fusions with the same results. Since this assay was carried out in a [cir^+] strain, it is not possible to tell whether Mcd1p can interact with one Rep protein alone in the absence of its partner. A [cir^0] host strain for the dihybrid assay is being constructed to address this question. Based on the results of the ChIP assays, we would expect no interaction between Mcd1p and Rep1p or Rep2p in the [cir^0] background.

We used the monohybrid assay to test *in vivo* the predicted association of Mcd1p with *STB* and to scrutinize the role of the Rep proteins in this interaction (Fig. 5.4). In this assay, the *STB* sequence provided the *UAS* for the *HIS3* reporter gene under the control of its basal promoter. If the Mcd1p-activation domain

hybrid protein can associate with *STB*, enhanced transcription of *HIS3* will ensue. Increased production of the His3 protein allows the tester strain to overcome growth inhibition by the His3p-specific inhibitor 3-aminotriazole (3-AT).

In a [cir^0] host strain, Mcd1p failed to interact with *STB*, yielding no better growth than the control strain containing the empty vector during 3-AT challenge (10 mM–50 mM; Fig. 5.4, compare columns 1 and 2), Under the same conditions, the positive control, Rep1p, conferred 3-AT resistance (Fig. 5.4, column 3). Previous experiments had demonstrated the ability of Rep1p to bind *STB* in a [cir^0] background. Co-expression of the Mcd1p fusion protein with Rep1p alone or Rep2p alone also failed to induce 3-AT resistance (Fig. 5.4, columns 4 and 5). The presence of all three proteins simultaneously in the same cell was required for growth at 25 mM and 50 mM 3-AT (Fig. 5.4, column 6). In a [cir^+] strain, the Mcd1p hybrid was active by itself, the Rep1 and Rep2 proteins being supplied by the native 2 micron circles (Fig. 5.4, column 8).

The agreement between the chromatin immunoprecipitation and monohybrid assays validates the inference that both Rep1p and Rep2p are mandatory for Mcd1p-*STB* association. The *in vivo* interaction between Mcd1p and the Rep proteins suggest that the latter are responsible for Mcd1p recruitment to the 2 micron plasmid. The involvement of all three components of the Rep-*STB* system in Mcdp1 (and likely the cohesin complex) recruitment suggests that this step is relevant to equipartitioning of the 2 micron plasmid.

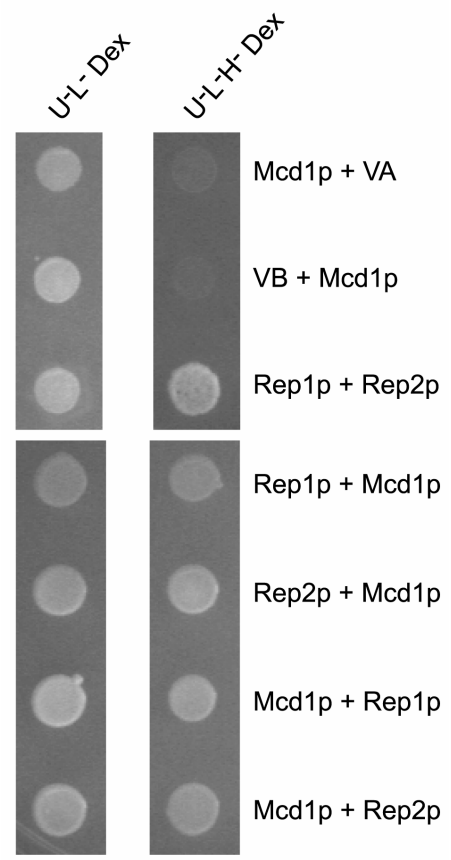


Figure 5.3 Interaction between Mcd1p and Rep proteins in the PJ (Philip James) dihybrid assay. *HIS3* was used as the reporter gene. VA stands for vector containing the Gal4p activation domain alone (not fused to Rep1p or its point mutant derivatives), while VB indicates the vector containing the Gal4p DNA binding domain alone. The protein listed at the left is the bait, and that at the right is the prey. The plasmids providing the bait and prey were maintained by keeping the selection for the *URA3* and *LEU2* markers harbored by them. U, L and H stand for uracil, leucine and histidine, respectively.

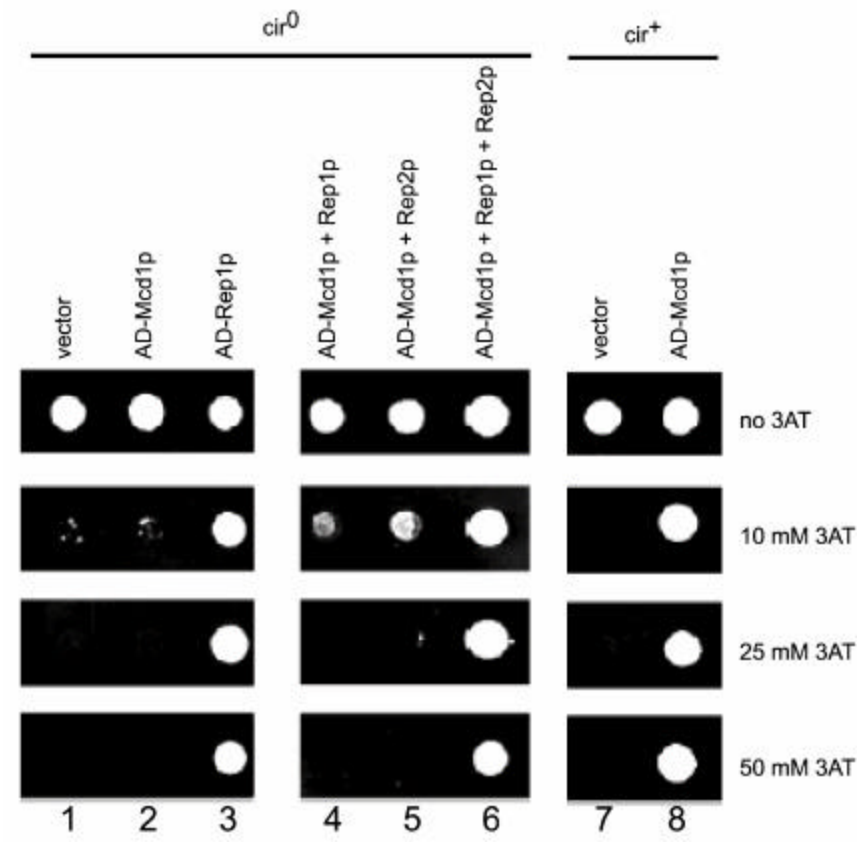


Figure 5.4 Interaction between Mcd1p and the *STB* element in yeast monohybrid assay. The growth of the strains in the presence of 10, 25 and 50 mM 3-AT is shown in rows two, three and four respectively. Plasmid constructs used in this test and the monohybrid strain background ([cir^+] or [cir^0]) are indicated on the top.

5.3.5 Binding of Rep1p and Mcd1p to *STB* as a function of cell cycle progression

The cohesin complex is loaded onto the chromosomes concomitant with DNA replication, providing a means to bridge duplicated sister chromatids (Carson and Christman, 2001). The cohesin mediated pairing lasts until the onset of anaphase when proteolytic cleavage of Mcd1p dissociates the complex. In published work (Mehta et al., 2002), we noticed that the timing of cohesin association and the life time of the cohesin-associated state during a cell cycle were identical for the 2 micron plasmid and the chromosomes. Is this because the recruitment of cohesin by the plasmid (which is duplicated during the S phase) is also replication dependent? Or does the timing simply reflect the assembly and disassembly of the cohesin complex during the cell cycle? And is there any cell cycle dependence for the binding of the Rep proteins to the *STB* locus? The ChIP experiments described below were carried out to address these issues.

A [cir^+] yeast strain containing the tagged *MCD1* gene was blocked in G1 with a factor, and then released from cell cycle arrest into pheromone-free growth medium. Samples of the culture were withdrawn at various times (see Fig. 5.5), and subjected to ChIP using antibodies directed to Mcd1p in one case and Rep1p in the other. Progression of the cell cycle was monitored by light microscopy. As noted previously, Mcd1p association with the *STB* locus or a chromosomal binding site was absent during the G1 phase (approxiamtely the 0-30 min.

interval), and was established at the start of the 'S' phase (45 min.). This pattern was repeated during the second cell cycle, as indicated by the absence of *STB* or the chromosomal site in the immunoprecipitate during the 90-105 min. interval. By contrast, the Rep1p was associated with *STB* during G1 (0-15 min.), followed by a brief period of dissociation during the G1-S interval (see the 30 min. time point) and subsequent re-establishment of the interaction at the onset of S phase (45 min.). Rep1p persisted on *STB* into G1 of the next cell cycle (90 min.), after Mcd1p had dissociated from it and from the chromosomal site. Once again, Rep1p was emptied from *STB* (105 min), and then reloaded on to it at the start of the S phase. Thus, the establishment of *STB* association with Rep1p and the cohesin complex is renewed synchronously during each cell cycle, even though the maintenance of this association is longer in the case of Rep1p. The overall results are consistent with the timing of *STB*-Rep protein interaction being the determinant of *STB*-cohesin interaction. In addition, the proteolysis of Mcd1p and disassembly of the cohesin complex during anaphase would clear *STB*, and set the stage for initiating a new cycle of cohesin association and dissociation.

Very similar results were obtained when the ChIP experiment was repeated using Rep2p antibodies (data not shown). The same cell cycle dependence for the association of Rep1p and Rep2p with *STB* is consistent with the two proteins being integral components of a high-order partitioning complex.

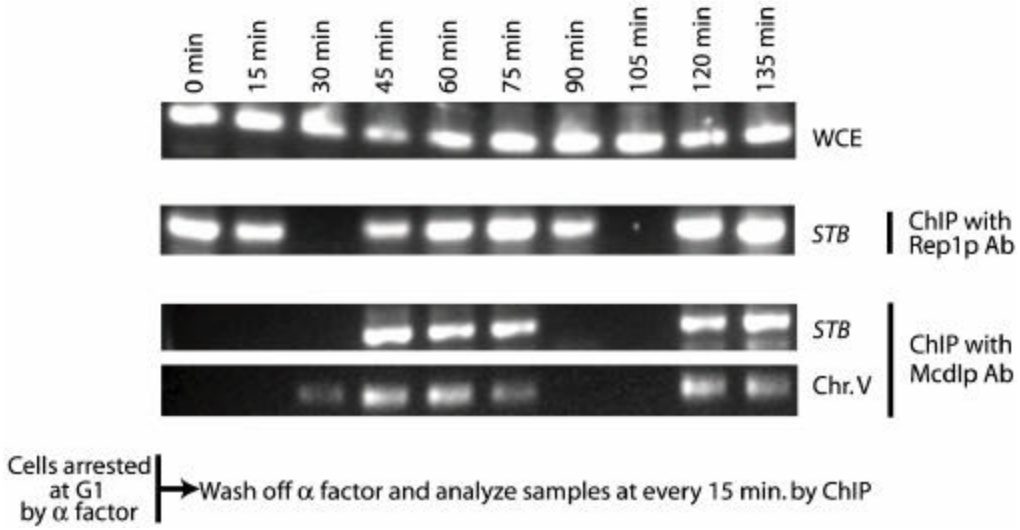


Figure 5.5 Cell cycle dependence of Mcd1p and Rep1p binding to the *STB* element. A brief scheme for the experiment procedure is outlined at the bottom. Time zero refers to the release of G1-arrested cells from a factor. Here and in other related figures, WCE stands for whole cell extract. The PCR reactions were done with primers specific to *STB* or to an Mcd1p binding site on chromosome V. The data shown here for WCE were obtained with *STB*-specific primers.

5.3.6 Association of inappropriately expressed Mcd1p with the *STB* locus

An experiment analogous to the one described in the previous section was carried out using a host strain containing an integrated copy of the tagged *MCD1* gene under the control of the inducible *GAL10* promoter (Fig 5.6). Cells were arrested in G1 with a factor in medium containing galactose so that Mcd1p expression occurred during the arrest. After removal of the a factor, they were allowed to resume the cell cycle in the presence of galactose. The difference in the sampling times between Fig. 5.5 and Fig. 5.6 reflects the increased generation time in galactose relative to glucose. The inappropriately expressed Mcd1p was able to bind *STB* during the G1 phase, whereas no binding to the chromosomal target was observed (Fig. 5.6). However, as was observed with the Rep proteins, the bound Mcd1p was stripped from *STB* in late G1, and the protein was rebound at the start of the S phase, in synchrony with its occupancy of the chromosomal sites. This neat temporal correlation between the *STB* association of the G1-expressed Mcd1p and that of the Rep proteins (Compare Fig. 5.6 to Fig. 5.5) is consistent with the Rep proteins mediating the plasmid recruitment of cohesin.

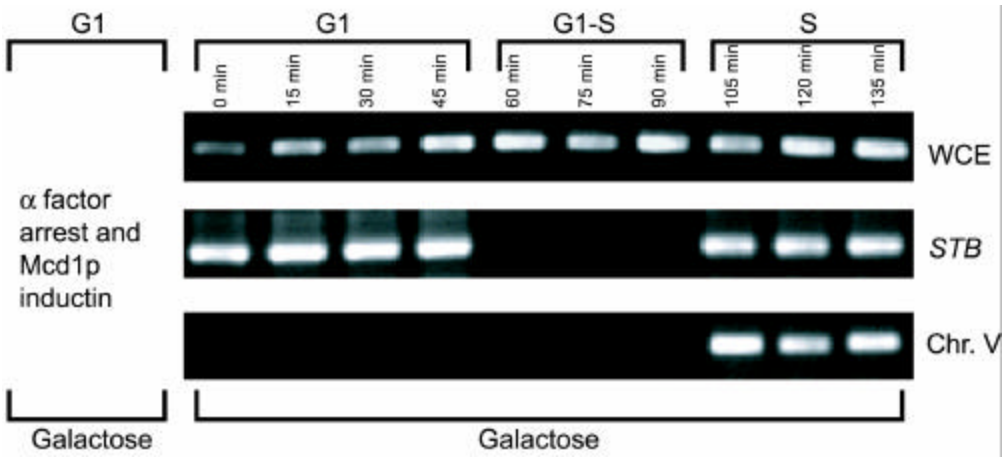


Figure 5.6 Binding of inappropriately expressed Mcd1p to the *STB* locus during cell cycle. Time zero refers to the release of G1-arrested cells from a factor. Cell phases (G1, G1-S and S) shown on the top are assigned according to observed cell morphology. And the fact that Mcd1p association with chromosomal sites is coincident with DNA replication. G1, prior to bud emergence; G1-S, period of bud emergence; S, period of bud elongation.

5.3.7 A potential role for the spindle in the association between cohesin and the 2 micron plasmid

One early observation regarding the 2 micron plasmid cluster was that nocodazole treatment results in a measurable decrease in the cohesiveness of the cluster as assayed by Z-series sectioning (Chapter 3; Velmurugan et al., 2000). More recent ChIP assays demonstrated that Mcd1p is not associated with *STB* in nocodazole treated cells, whereas there is no effect of the drug on Mcd1p-chromosome association (Fig. 5.7, lanes 2 and 3). Similar experiments also showed that the drug does not interfere with the interaction between the Rep proteins and *STB* (Fig. 5.7, lanes 4-7). An obvious question raised by this result is whether the depletion of cohesin from *STB* would lead to defective plasmid partitioning during cell division. If cohesin dependent pairing of plasmid clusters is essential for equal segregation, uneven distribution of plasmids to the daughter cells would be the expected result. However, since chromosome segregation requires the integrity of the spindle, the drug has to be removed to allow spindle reassembly before plasmid stability can be assayed by standard procedures at the population level.

5.3.8 Microtubule depolymerization, plasmid cohesion and segregation

The following experiment was done to examine whether nocodazole treatment

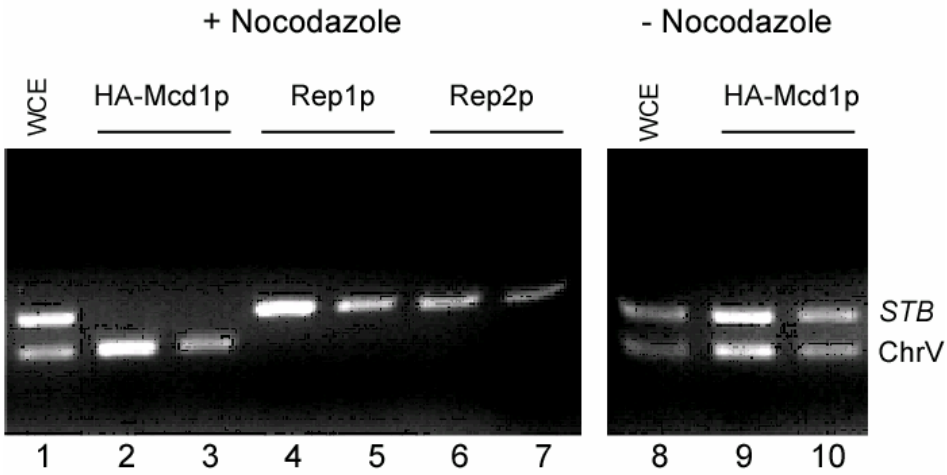


Figure 5.7 Effect of nocodazole on Mcd1p association with the *STB* element. In the paired set of lanes (2 through 7, 9 and 10), the right hand lanes represent half the amount of immunoprecipitated samples relative to the corresponding left hand lanes.

for varying times has any effect on plasmid partitioning. G1-blocked [cir^+] cells harboring an *STB* containing test plasmid were washed free of a factor and resuspended in fresh growth medium for 15 min at 30 ºC. At this time (referred to as time zero) nocodazole was added to the culture. Aliquots were removed at zero time and at fifteen-minute intervals thereafter up to three hours. After washing off nocodazole, each sample was plated on appropriate plates at different dilutions to estimate the mitotic stability of the plasmid (Table 5.2). There was no detectable difference in plasmid stability between treated and untreated cells.

There are two possible explanations for the outcome from the above experiment (Table 5.2). One is that cohesin is not directly involved in plasmid partitioning. The other is that cohesin may have reassociated with the plasmid after nocodazole has been removed. To investigate these possibilities further, a nocodazole recovery experiment was performed. The yeast strain used here is MJY146 (see Materials and Mathods, Chapter 2; In this strain, Mcd1p is tagged with 3HA and expressed from its native chromosome locale, and one copy of YFP-tubulin gene is integrated into chromosomal *URA3* site). Synchronized cells were treated with nocodazole for two hours after release from G1 arrest, washed free of the drug (time zero) and transferred to growth medium. Cell aliquots were sampled at time zero and every fifteen minutes thereafter by chromatin immunoprecipitation and fluorescence microscopy for tubulin (Fig. 5.8). The striking outcome was that cohesin-*STB* association was restored after nocodazole

Table 5.2 Stability of an *STB* plasmid after nocodazole treatment followed by removal of the drug

Time (min) with nocodazole treatment	Mitotic stability (%)	Time (min) with nocodazole treatment	Mitotic stability (%)
0 min	114.5	90 min	94.3
15 min	97.1	105 min	89.5
30 min	105.3	120 min	97.5
45 min	97.1	135 min	97.3
60 min	100.0	150 min	94.1
75 min	100.9	165 min	100.6
		180 min	106.7

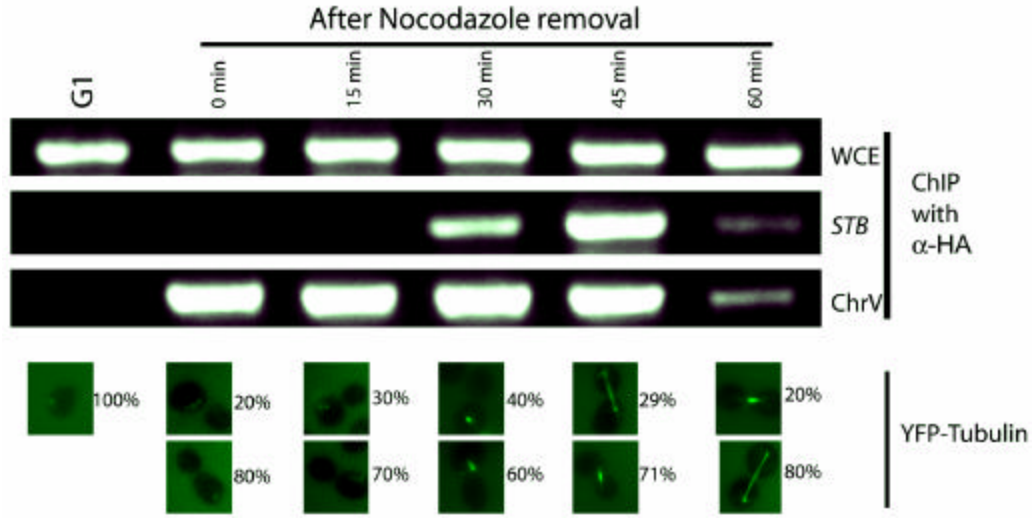


Figure 5.8 Association of Mcd1p with its chromosomal binding site and the *STB* element after nocodazole treatment and removal of the drug. After cells were released from G1, nocodazole was added to the culture for two hours before being washed off. Time zero refers to the time of release from nocodazole. Representative YFP images of the tubulin at the different time points are shown at the bottom. The values beside each image indicate the fraction of cells that display the indicated spindle pattern at a particular time point.

removal, and the timing of this recovery was strongly correlated with the reestablishment of the spindle. The lack of Mcd1p occupancy of either *STB* or the chromosomal target locus at the 60 min. time point (Fig. 5.8) was consistent with the anaphase associated degradation of Mcd1p.

The above results clearly indicate that the presence of the mitotic spindle is required for the recruitment of cohesin to the 2 micron plasmid, although the mechanism by which the spindle facilitates this event is unclear. Since DNA replication is not delayed in the absence of the spindle, we believe that binding of cohesin to *STB* need not occur concomitant with plasmid duplication. This is an important departure from the mode of cohesin binding to the chromosomal sites. It is possible that the replicated plasmids form two clusters and the role of cohesin may be simply to bridge the clusters rather than bridge each pair of sister molecules as is the case with sister chromatids.

5.4 Discussion

The studies reported in this chapter shed light on what types of host encoded proteins may influence 2 micron plasmid partitioning. The requirement of such factors was suggested by the isolation of Rep1p mutants that fail to support plasmid stability even though these mutations have no effect on Rep1p-Rep2p interaction or Rep1p-*STB* interaction. In vivo screens revealed chromosomally encoded proteins that interact with the plasmid partitioning

system, and they have been independently identified to be involved in chromosome partitioning. Since one of these proteins, Brn1p, is a condensin subunit, we pondered the requirement of the condensin and cohesin complexes in plasmid segregation. However, the apparent lack of specificity in the binding of codensin to DNA, narrowed our focus to cohesin. The results presented here with the cohesin subunit Mcd1p are in agreement with the general model in which plasmid and chromosome partitioning steps are linked in some manner.

5.4.1. *BRN1* and *FUN30* in plasmid segregation

The *BRN1* and *FUN30* gene products interact with the plasmid stability system at the level of the Rep proteins. While Brn1p interacts with Rep1p and Rep2p independently, Fun30p interacts with only Rep1p directly. Both proteins interact with *STB* indirectly through the mediation of the Rep proteins. At this time, we do not have evidence for these proteins being functional in 2 micron plasmid partitioning. However, their requirement for chromosome maintenance suggests that the possibility of a similar role in plasmid maintenance can not be ignored. Furthermore, we have identified a Rep1p mutant that interacts with Rep2p and *STB* but not with Brn1p, and is unable to support the stability of a 2 micron reporter plasmid.

The interaction of Fun30p with the plasmid partitioning system is significant in that this protein contains a strong motif characteristic of the *SNF2*

transcriptional regulators involved in chromatin remodeling (Ouspenski et al., 1999). A recent report (Wong et al., 2002), has clearly demonstrated the requirement for Rsc2p, a component of one of the chromatin remodeling complexes in yeast, for the stable propagation of the 2 micron plasmid. The chromatin organization of *STB* in the *rsc2* deletion background is not normal, and the Rep protein-*STB* association appears to be affected as well. It seems plausible that other chromatin remodeling proteins such as Fun30p may also contribute to the functional organization of *STB*. In this context, it must be noted that we had earlier identified the Shf1 protein (for 'STB binding host factor) whose absence causes a small but consistent increase in the loss rate of the 2 micron plasmid (Velmurugan et al., 1998). The same gene product was revealed in a separate overexpression screen that scored the loss of a reporter minichromosome (Ouspenski et al., 1999). The gene was named *CST6* by these authors. The sequence of *CST6/SHF1* indicates a consensus CREB motif common to the ATF/bZIP family of transcriptional regulatory proteins. Thus, the high-order organization as well as the transcriptional status of the *STB* region is likely to be important in the assembly of the active plasmid partitioning complex. It is interesting to note that the *STB* region proximal to the 2 micron replication origin is normally free of transcriptional activity. One of the major plasmid transcripts (1650 n.t.) is terminated just at the border of this *STB* region (Sutton and Broach, 1985).

5.4.2 Cohesion, microtubule depolymerization and plasmid partitioning

The most interesting, and perhaps intriguing, findings reported here concern the association of the cohesin subunit Mcd1p with the *STB* locus in a Rep protein dependent manner. A series of results suggest that it is the whole cohesin complex that is recruited to the plasmid. Several of the details of this association inferred by ChIP assays (Mehta et al., 2002) have been confirmed by in vivo genetic assays as well. We have shown that the association of cohesin with *STB* is clearly distinct from that with chromosomal binding loci. For example, Mcd1p inappropriately expressed in G1 can be detected at *STB*, but not at a chromosomal site, by ChIP. *STB* does not share sequence similarities with cohesin binding sites on the chromosomes. And obviously, the Rep proteins are dispensable in the chromosomal recruitment of cohesin. Yet the timing of cohesin association and dissociation are well synchronized between the chromosomes and the 2 micron plasmid. The factors responsible for this synchrony are (1) the timing of Mcd1p expression, (2) the recycling of the Rep proteins on the *STB* DNA and (3) the timing of Mcd1p degradation. The temporal sequence of Rep protein association with *STB* and dissociation from it are nicely optimized for the plasmid to feed into the temporal program established for the chromosomal cohesin association-dissociation cycle.

Assuming that the yeast cohesin complex plays fundamentally similar roles in the partitioning of yeast chromosomes and the 2 micron plasmid, one or

more segregation models can be considered. It is possible that cohesin facilitates pairing between the two duplicated plasmid clusters that, in turn, are tethered to a pair of sister chromatids. The coincident dissolution of the cohesin bridge between the sister chromatids and the plasmid clusters would dispatch each cluster in opposite directions in association with the chromosomes. The plasmid-chromosome attachment could be mediated by cohesin itself or through other factors. If the former mode of tethering is correct, there must be some mechanism to postpone Mcd1p cleavage within this tether until after segregation has been completed. Another possibility is that the two post-replication plasmid clusters are bridged by the cohesin complex but are not tethered to chromosomes. Upon disassembly of cohesin, each unpaired plasmid cluster moves to opposite cell poles without assistance from the chromosomes. This movement may be mediated by spindle attachment (a spindle associated motor protein could be involved), by an active transport system unrelated to the spindle or by association with a subcellular entity that is evenly partitioned at cell division.

5.4.3 Unanswered questions

The earlier results concerning spindle integrity and the compactness of the plasmid cluster (Chapter 3; Velmurugan et al., 2000) as well as the present ones demonstrating a potential link between the spindle and cohesin recruitment by the plasmid appear to suggest some functional relation between these two

observations. Admittedly, we do not know how to make this connection at this time. Is the spindle in someway responsible for spatially localizing the plasmid cluster (in the vicinity of kinetochores?) so that it has direct access to the cohesin complex? Or is cohesin mediated pairing of plasmid clusters and their attachment to the mitotic spindle coupled events in a segregation pathway that is independent of the chromosomes? Answers to these questions should be insightful but require future in-depth investigations.

CHAPTER 6

The *STB* Locus: Merely a Recruitment Site for the Rep1 and Rep2 Proteins?

6.1 Abstract

1. The 2 micron stability system consisting of the *STB* locus and the Rep1 and Rep2 proteins can be reconstituted with reasonable efficiency by an alternative system. In the latter, the *STB* is substituted by four copies of the LexA operator DNA, and the native Rep proteins are replaced by LexA-Rep1p and LexA-Rep2p.

2. Surprisingly, the LexA-Rep/LexA operator system provides equivalent stability when LexA-Rep2p alone is supplied or both LexA-Rep1p and LexA-Rep2p are supplied.

3. As has been observed for the Rep/*STB* system, the positioning of the operator repeats with respect to the plasmid origin can significantly influence the efficiency of the LexA-Rep/LexA operator mediated plasmid partitioning.

4. We interpret the above results in terms of the 'recruitment model' in which the critical event in plasmid partitioning is the association between Rep2p and the partitioning locus. When *STB* provides this locus, Rep1p is an essential

accessory factor in partitioning; when LexA operators serve as the partitioning locus, Rep1p is dispensable.

6.2 Background

The cumulative results summarized in Chapters 3-5 suggest that the plasmid segregation apparatus is a multi-component molecular assembly involving two plasmid proteins and possibly multiple host proteins. The Rep1 and Rep2 proteins appear to physically deliver plasmid molecules to the partitioning machinery. The observed interaction between the Rep proteins and between Rep proteins and host encoded proteins appear to fit nicely the paradigm of 'molecular recruitment' (popularized by Ptashne in the context of transcription; Ptashne and Gann, 1997). Broadly, recruitment refers to an increase in the lifetime of the productive interaction between the RNA polymerase enzyme and a cognate promoter sequence via a series of protein-protein and DNA-protein contacts mediated by transcription factors, 'activator' and 'co-activator' proteins as well as *UAS* and enhancer DNA elements. Viewed from this perspective, the partitioning problem reduces to recruiting a DNA sequence (perhaps the *STB* locus of the 2 micron circle) distributed on different plasmid molecules to localized cognate receptor regions (the partitioning center). Stable partitioning would result from the enhanced half-life of the 2 micron plasmid in this spatially restricted state. This active state would be short-lived for *ARS* plasmids or 2 micron circle derivatives lacking the partitioning system, thereby increasing the probability of their missegregation.

The experimental strategies described below are designed within the general realm of the recruitment model.

6.3 Rationale of the experiments

By analogy to Ptashne's transcription model, we consider two possible roles for the *STB* locus: (a) it serves simply as a recruitment site for Rep1p and Rep2p, and these proteins then function in partitioning; (b) aside from serving as the binding site for the Rep proteins, *STB* plays a second distinct role in partitioning. If the first possibility is right, we should be able to obtain normal levels of plasmid stability when the Rep proteins are recruited to the plasmid by an *STB*-independent mechanism. For the experiments described below, we constructed reporter plasmids lacking *STB* but containing the 2 micron origin and four copies of the *E. coli* LexA operator sequence. The ability of LexA-Rep1p and/or LexA-Rep2p hybrid proteins to support their stable maintenance was then assayed.

6.4 Results

6.4.1 LexA-Rep1p and LexA-Rep2p hybrid proteins can replace the corresponding wild type proteins in 2 micron circle segregation

The design for testing the recruitment model is based on the premise that the fusion proteins formed between LexA and Rep1p and LexA and Rep2p can carry out the functions of the native Rep proteins after they have been loaded on to a target plasmid. To verify this premise, the LexA protein was fused to the N-terminus of Rep1p in one case and the N-terminus of Rep2p in the other. Each of the fusion cassettes was placed in a yeast expression vector, such that the Rep1p hybrid or the Rep2p hybrid was expressed from the *ADH1* promoter. In the control experiment, wild type Rep1p and Rep2p were expressed from the endogenous 2 micron plasmid molecules present in a [cir^+] host strain.

Three *STB*-containing plasmids were used as reporters in these assays, and are designated as cp20 (pSTB), cp21 (pSTB-REP1), cp22 (pSTB-REP2). The first one did not harbor either the *REP1* or *REP2* locus, and was the substrate for testing the consequence of co-expressing LexA-Rep1p and LexA-Rep2p in a [cir^0] background. The stability of this plasmid in a [cir^+] strain provided the reference value for the normal activity of Rep1p and Rep2p. The other two, as indicated by their names, supplied either native Rep1p or native Rep2p. And they served to report on the functionality of LexA-Rep1p and LexA-Rep2p.

Table 6.1 LexA-Rep proteins are functional in the maintenance of 2 micron plasmids

Strain	Assay number	Rep protein (s) provided (source)	LexA-Rep protein (s) provided (source)	Test Plasmid	Mitotic Stability (%)
CRY1-cir^0	1	Rep2p (cp22)	LexA (pBTM116)	cp22	13.1 ± 4.36
	2	Rep2p (cp22)	LexA-Rep1p (pBTM116)	cp22	90.7 ± 20.6
	3	Rep1p (cp21)	LexA (pBTM116)	cp21	20.5 ± 11.4
	4	Rep1p (cp21)	LexA-Rep2p (pBTM116)	cp21	96.6 ± 8.52
	5	/	LexA-Rep1p (pEG202) & LexA-Rep2p(pBTM116)	cp20	54.6 ± 11.8
CRY1-cir$^+$	6	Rep1p & Rep2p (native 2 micron)	/	cp20	84.0 ± 10.2

The mitotic stability data shown in Table 6.1 were obtained from 10 independent transformants harboring the appropriate plasmids in each experimental group. The experimental procedure for the stability assay has been outlined under 'Materials and Methods' (Chapter 2) and elsewhere in this thesis. It is clear from Table 6.1 that both LexA-Rep1p and LexA-Rep2p are functional in plasmid maintenance. When either one of the hybrid Rep proteins was paired with its wild type partner Rep, the mitotic stability of the *STB*-containing plasmid was the same as (or even better than) that observed when both the Rep proteins were wild type (Table 6.1, rows 2 and 4 and 6). When LexA-Rep1p and LexA-Rep2p were partnered, the stability of cp20 (pSTB) dropped somewhat (to nearly 55%; row 5, Table 6.1), but was till markedly higher than that obtained with the LexA protein as the control (rows 1 and 3, table 6.1). Therefore, the LexA-Rep protein fusions do support *STB* plasmid stability, even though their combination is less active than that of their wild type counterparts.

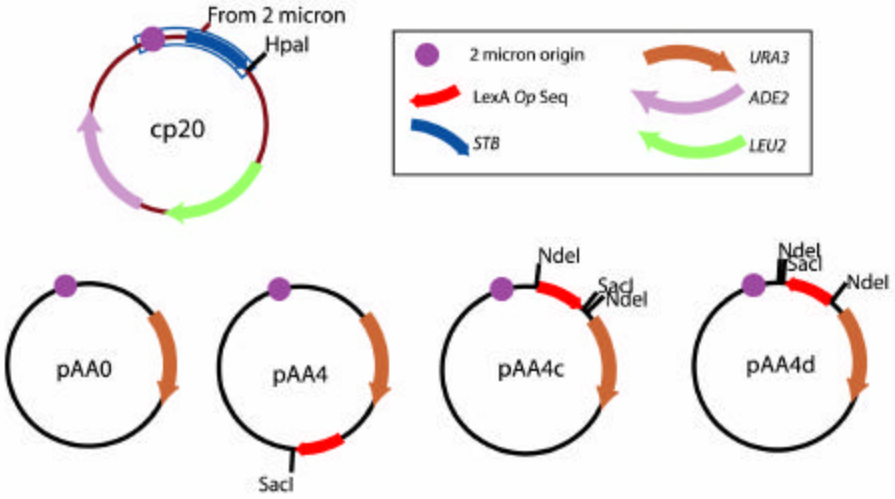
6.4.2 LexA-Rep hybrid proteins can stabilize plasmids lacking *STB* but containing the 2 micron circle origin and LexA operator repeats

To test the prediction from the recruitment model, two similar reporter plasmids, both lacking *STB*, were constructed: pAA0 (pORI-OP0) and pAA4 (pORI-OP4). The former served as the control, and harbored the 2 micron circle origin plus the *URA3* gene, which provided the marker for the stability assay. The

latter (the test plasmid), contained in addition, 4 copies of the LexA operator sequence cloned approximately 2.2 kbp from the origin. The plasmids are schematically represented in Table 6.2. The LexA-Rep proteins, expressed from the *ADH1* promoter, were supplied from 2 micron circle based expression plasmids with appropriate markers (*HIS3* in one and *TRP1* in the other). The stability assay was carried out in a [cir^0] host strain containing three plasmids, the reporter plasmid plus the expression plasmids for the hybrid Rep proteins. As shown in Table 6.2, the mitotic stability of pAA4 (pORI-OP4) was increased nearly 1.6 fold compared to that of the control pAA0 (pORI-OP0) plasmid when the LexA-Rep fusion proteins were supplied. By contrast, pAA4 (pORI-OP4) and pAA0 (pORI-OP0) showed the same low stability (about 15 %; Table 6.2) in the [cir^+] strain (expressing native Rep1p and Rep2p).

The data in Table 6.2 suggest that the LexA-Rep hybrid proteins do elevate the stability of a plasmid harboring repeated LexA operator DNA. The magnitude of the effect, though significant, is not particularly striking. Furthermore, it is known that the spacing between the *STB* locus and the 2 micron circle origin can also significantly alter its efficiency in plasmid partitioning. As described in Chapter 1, the origin proximal portion of *STB* (containing the repeated elements) is transcriptionally silent in the 2 micron circle (Murray and Cesareni, 1986). For these reasons, we have extended the above experiment using

Table 6.2 Mitotic stabilities of LexA operator-containing plasmids



Strain	Assay number	Vector 1 (pEG202)	Vector 2 (pBTM116)	Test Plasmid	Mitotic Stability (%)
CRY1-cir^0	1	LexA-Rep1p	LexA-Rep2p	pAA0	18.2 ± 3.5
	2	LexA-Rep1p	LexA-Rep2p	pAA4	29.8 ± 7.8
	3	LexA-Rep1p	LexA-Rep2p	pAA4c	36.0 ± 9.8
	4	LexA-Rep1p	LexA-Rep2p	pAA4d	43.2 ± 11.3
	5	LexA-Rep1p	LexA-Rep2p	cp20	54.6 ± 11.8
CRY1-cir$^+$	6	LexA	LexA	pAA0	17.8 ± 5.8
	7	LexA	LexA	pAA4	14.1 ± 7.4

reporter plasmids in which the LexA operator repeats were cloned in the vicinity of the plasmid origin (see below).

6.4.3 The efficiency of LexA operators as a plasmid partitioning locus is dependent on their proximity to origin

We constructed two alternative versions of pAA4 (pORI-OP4) in which the LexA operator was moved to approximately 450 bp from the origin, a distance compatible with the *STB* to *ORI* spacing in the 2 micron circle. These derivatives, pAA4c (pORI-OP4-1) and pAA4d (pORI-OP4-2), differ only in the relative orientations of the DNA segment containing the operators with respect to the origin. The mitotic stability of these plasmids in the presence of the LexA-Rep proteins was increased 2 to 2.5 fold relative to pAA4 (pORI-OP4). The stability of pAA4d (pORI-OP4-2) approached that of an *STB*-plasmid (pSTB) supplied with LexA-Rep1p and LexA-Rep2p (Table 6.2). In other words, the operator repeats and the *STB* locus were virtually equivalent in their efficiency when partitioning was mediated by the hybrid Rep proteins.

Based on the sum of the results obtained with the different operator containing plasmids, we argue in favor of the recruitment model: that is, once the Rep proteins have been stably associated to the plasmid, the subsequent partitioning functions follow. The data also indicate that there is a positional component to this recruitment, as revealed by differential plasmid stabilities

conferred by changes in the distance between the operators and the replication origin.

6.4.4 LexA-Rep2p together with wild type Rep1p can stabilize a LexA operator-containing plasmid

When a plasmid is maintained via the LexA operator acting as the partitioning locus, do both the Rep1 and Rep2 protein have to be in the hybrid form as LexA fusions? Or will combinations of LexA-Rep1p/Rep2p or Rep1p/LexA-Rep2p suffice? Since the Rep proteins interact with each other, it is not unlikely that only one protein needs to be anchored at the operator site in order to recruit its partner.

We examined the stability of pAA4 (pORI-OP4) in a [cir^0] strain expressing LexA-Rep1p together with wild type Rep2p (supplied by an *STB*-plasmid harboring *REP2* under its native promoter) or LexA-Rep2p together with Rep1p (provided by an *STB*-plasmid carrying *REP1* driven by its own promoter). One of the two combinations, namely, LexA-Rep2p/Rep1p was able to increase the stability of pAA4 (pORI-OP4) (compare rows 3 and 4, Table 6.3). By contrast neither combination had any effect on the stability of the control plasmid pAA0 (pORI-OP0; lacking the LexA operators).

Two interpretations are consistent with the above observations. In one, plasmid stability is dependent on the recruitment of both Rep1p and Rep2p to the

Table 6.3 Mitotic stability mediated by LexA operators in the presence of a single LexA-Rep protein

Strain	Assay Number	Rep protein (s) provided (vector)	LexA-Rep protein (s) provided (vector)	Test Plasmid	Mitotic Stability (%)
CRY1-cir^0	1	Rep2p (cp22)	LexA-Rep1p (pEG202)	pAA0	21.7 ± 5.2
	2	Rep2p (cp22)	LexA-Rep1p (pEG202)	pAA4	25.3 ± 6.7
	3	Rep1p (cp21)	LexA-Rep2p (pBTM116)	pAA0	25.9 ± 5.7
	4	Rep1p (cp21)	LexA-Rep2p (pBTM116)	pAA4	49.0 ± 8.1

pBTM116, pEG202, cp21 and cp22 are *STB* plasmids

operator site. However the assembly of the functional partitioning complex requires an ordered pathway in which Rep2p interaction with the plasmid precedes the entry of Rep1p. In the alternative interpretation, stable association between Rep2p and the plasmid is sufficient to achieve plasmid stability, and Rep1p is completely dispensable. In the normal situation, with *STB* serving as the partitioning locus, the presence of Rep1p may be essential to establish stable Rep2p interaction.

6.4.5 LexA-Rep2p by itself is sufficient to stabilize a plasmid containing LexA operators

In the previous set of stability experiments, the native Rep1p or Rep2p was supplied by an *STB*-containing plasmid. We wished to repeat the experiments in a [cir^0] background with no *STB*-containing plasmid included in the assay system. In this manner we could eliminate potential titration effects arising from the partitioning of a LexA-Rep hybrid protein between the operator on the one hand and *STB* on the other. The stability assays with pAA0 (pORI-OP0) and pAA4 (pORI-OP4) were repeated by expressing the LexA-Rep hybrids from a *TRP1-CEN* plasmid. Since this plasmid contained the bidirectional *GAL1-GAL10* promoter, it was possible to simultaneously induce one of the Rep proteins in its native form and the other as the LexA hybrid.

As shown in Table 6.4, the LexA-Rep2 protein by itself was capable of mediating the maintenance of pAA4 (pORI-OP4) at moderately high stability (Table 6.4, row 6). There was only a minor additional effect, if at all, due to Rep1p (Table 6.4, row 8). The presence of the LexA operator on the reporter plasmid was essential for LexA-Rep2p induced stability. Neither LexA-Rep2p alone (Table 6.4, row 2) nor the LexA-Rep2p/Rep1p combination (Table 6.4, row 4) had any effect on the stability of pAA0 (pORI-OP0). When dextrose was the carbon source, the stability of pORI-OP4 dropped to approximately 4% in the presence of the *CEN*-plasmid harboring LexA-*REP2* (Table 6.4, row 5) and to approximately 9% in the presence of the *CEN*-plasmid harboring LexA-*REP2* as well as *REP1* (Table 6.4, row 7). Thus, the higher stability of pORI-OP4 was absolutely dependent on the galactose mediated induction of LexA-Rep2p. The stability values for pORI-OP0 in dextrose were comparable to those for pORI-OP4 under similar conditions (Table 6.4, row 1 and 3).

The difference in the basal stability values of the test plasmids between this assay (less than 10%; Table 6.3) and the assays presented in Tables 6.1 and 6.2 (as high as 25%) is probably due to the difference in the manner in which the LexA-Rep proteins were supplied in these experiments, via *STB* containing plasmids in one case and *CEN* plasmids lacking *STB* in the other. The stabilities of the *CEN* and *STB* plasmids are quite different. Unlike the *CEN* plasmids, the *STB* plasmids have to utilize the Rep proteins (or the LexA hybrid versions of the

Table 6.4 Mitotic stability mediated by LexA operators in the presence of a single LexA-Rep protein expressed from a *CEN* plasmid under the *GAL* promoter

Strain	Assay number	Carbon Source	Rep proteins provided from CEN vector		Test plasmid	Mitotic Stability (%)
			Rep1p	LexA-Rep2p		
MJY92 (cir^0)	1	dextrose	/	LexA-Rep2p	pAA0	4.60 ± 1.84
	2	galactose	/	LexA-Rep2p	pAA0	1.21 ± 0.35
	3	dextrose	Rep1p	LexA-Rep2p	pAA0	8.88 ± 2.56
	4	galactose	Rep1p	LexA-Rep2p	pAA0	2.48 ± 2.26
	5	dextrose	/	LexA-Rep2p	pAA4	4.16 ± 1.73
	6	galactose	/	LexA-Rep2p	pAA4	40.3 ± 11.6
	7	dextrose	Rep1p	LexA-Rep2p	pAA4	8.43 ± 4.95
	8	galactose	Rep1p	LexA-Rep2p	pAA4	55.1 ± 8.38

The plasmids pAA0 and pAA4 are the same as pORI-OP0 and pORI-OP4, lacking LexA operators or containing four copies of it, respectively. The LexA-Rep2p alone or LexA-Rep2p and Rep1p together were expressed from *CEN*-plasmids by the *GAL10* promoter and the *GAL1-GAL10* bidirectional promoter, respectively.

Rep proteins) for their own partitioning. Thus, in the earlier experiments, the stability of the reporter plasmids was biased by the selection of a subpopulation that retained the *STB* plasmids supplying the Rep proteins in native/hybrid form.

6.5 Discussion

6.5.1 The recruitment model for plasmid partitioning

The results presented in this chapter are consistent with a recruitment model for the mechanism of action of the Rep/*STB* system in plasmid partitioning. The fundamental tenet of this model, initially proposed to explain induction of transcription by RNA polymerase (Ptashne and Gann, 1997), is that a given physiological function is dependent only on achieving a critical concentration and a finite half-life of the active components at a particular genomic locale. The particular mechanism by which the assembly is mediated is irrelevant. Or, more than one type of intermolecular interaction may be utilized to obtain the same functional reaction complex. We have shown here that the nature of the partitioning locus *per se* is not critical in plasmid partitioning, provided the requisite protein(s) can be targeted to it.

Initially, we showed that the *STB* sequence can be replaced with four repeats of the LexA operator to obtain near normal plasmid stability when the Rep proteins are supplied as fusions to the LexA protein. Subsequently, we found that

the same result is also produced by the LexA-Rep2p/Rep1p combination of proteins but not by the LexA-Rep1p/Rep2p combination. Finally, it became apparent that LexA-Rep2 alone can mediate stability when the LexA operators provide the partitioning locus on the plasmid. This outcome was rather unexpected, since *STB* mediated partitioning of the 2 micron circle requires the simultaneous presence of both Rep1p and Rep2p. We had shown earlier that Rep1p can bind to *STB in vivo* in the absence of Rep2p and vice versa.

We can still reconcile the apparently conflicting observations on the role of Rep1p in plasmid stability within the frame work of the recruitment model. We would argue that the functional entity for partitioning is only Rep2p. However when the recruitment locus is *STB*, Rep1p is required as an accessory factor to build up the requisite local concentration of Rep2p and to confer the appropriate half-life on the *STB*-Rep2p complex. In other words, in the Rep-*STB* system, the price for the functional recruitment of Rep2p is the sum of the binding energies derived from Rep1p and Rep2p contacts with *STB* (and perhaps from Rep1p-Rep2p contacts as well). In the LexA operator/LexA-Rep2p system, the binding energy from the strong repressor-operator interaction is likely more than enough to satisfy the functional recruitment of Rep2p. Hence the contribution from Rep1p is no longer required.

6.5.2 Potential arguments against the recruiting model

In interpreting our results, we have tacitly assumed that the mode of plasmid partitioning by the Rep/*STB* system and the LexA-Rep2p/lexA operator system are mechanistically the same. Although the assumption is quite reasonable, there are certain caveats to it that need to be spelled out. Evidence in literature suggests that plasmids containing a replication origin can be maintained in yeast by more than one mechanism. For example, the stability of *ARS* plasmids can be increased by the inclusion of telomeric sequences or the mating type silencing sequences in their genomes (Kimmerly and Rine, 1987; Longtine et al., 1992). Plasmid stabilization by the telomeric sequences requires the Rap1 protein (Longtine et al., 1993) and by the silencer requires the Sir4 protein (Ansari and Gartenberg, 1997). It is possible that complexes for transcriptional silencing (including the Rap1 or Sir4 protein) localize their target chromatin regions to nuclear sites that are partitioned more or less equally at cell division. More recently, a completely heterologus partitioning mechanism utilizing the Epstein-Barr virus based stability system has been reconstituted in yeast (Kapoor et al., 2001). Here the EBV segregation element (FR) provides the *cis*-acting partitioning locus, and the EBNA1 protein in conjunction with the human EBP2 protein provides the *trans*-acting functions. By analogy to the EBV episomes in mammalian cells, it is presumed that improved stability of FR-containing *ARS*-plasmids is due to their tethering to chromosomes.

Thus, if the plasmid stability mediated by the LexA-Rep2p/LexA operator system follows a mechanism distinct from that of the Rep/*STB* system, the interpretation of our results in terms of the recruitment model would be invalid.

6.5.3 Questions to be addressed

1. As already noted, we do not know for certain whether the Rep/*STB* system and the LexA-Rep2p system follow the same pathway for plasmid maintenance. According to the current working model, the Rep1p and Rep2p proteins act in concert to recruit the yeast cohesin complex to the *STB* locus. Cohesin assembly at *STB* and subsequently its disassembly at the anaphase transition are thought to be important for the equal partitioning of 2 micron circles between the daughter cells. One important question to be answered is: Can LexA-Rep2p recruit the yeast cohesin complex to the LexA operator DNA? The experimental tools and reagents to perform the relevant experiments are available.

2. If the recruitment model is correct, can we device an alternative mode of Rep protein recruitment to organize an active partitioning complex? We intend to replace the *STB* elements by multiple repeats of the recognition site (*loxP*) for the Cre recombianse. Stability of the plasmids harboring $(loxP)_n$ will be assayed in the presence of Cre-Rep1p alone, Cre-Rep2p alone and the two hybrid proteins together. Since the arrangements of the *loxP* sites are good for Cre binding but not for DNA breakage by Cre, there will be no interference due to recombination. The

experiment can also be done using the catalytically inactive mutant protein Cre(Phe). The Cre protein has a relatively high affinity for *loxP* (K_d of approximately 10^{-9}M), although not as high as that of repressor-operator interactions (K_d of approximately 10^{-11}M; Ringrose et al., 1998). Assuming that the Cre-Rep/*loxP* system works, it would be interesting to see whether plasmid stability requires Cre-Rep2p alone or both Cre-Rep1p and Rep2p.

3. So far, partitioning models have postulated that the association of both Rep1p and Rep2p with *STB* are important for stable maintenance of the 2 micron circle. It was a surprise to note that LexA-Rep2p by itself was able to provide significant stability to a plasmid containing the LexA operator repeats. As explained earlier, in the normal Rep-*STB* system, Rep1p may provide the additional binding energy required for the stable association of Rep2p with *STB*. Recall that Rep2p can bind *STB in vivo* (results from monohybrid assays) even in the absence of Rep1p. This raises the question: If we increase the number of the consensus *STB* elements (from the normal six to say 12 or 18), can Rep2p/*STB* provide plasmid stability without assistance from Rep1p? The requisite experiments are straightforward to perform.

EPILOGUE

What has been accomplished

The purpose of the work presented here was to begin to understand the mechanisms by which the yeast plasmid 2 micron circle persists with almost chromosome-like stability without conferring any obvious advantage to its host. The results summarized in this thesis indicate that we have made progress in this direction.

The organization of the multicopy plasmid in the yeast nucleus as a cohesive cluster and its segregation also as a cluster provides a reasonable basis for the evolution of an active plasmid partitioning system. The localization of the plasmid clusters in the close vicinity of spindle poles, their dynamics during the cell cycle and the similarity in the segregation kinetics of plasmids and chromosomes suggest direct or indirect links between their partitioning mechanisms. Consistent with this idea, mutations that cause missegregation of chromosomes have an identical effect on the 2 micron plasmid. Strikingly, in these mutants, plasmids missegregate in tandem with the bulk of the chromosomes.

The efficient and more or less equal segregation of plasmids at cell division is mediated by a tripartite DNA-protein system: the Rep1 and Rep2 proteins coded for by the plasmid and the *STB* locus situated in the proximity of

the replication origin. The mutational studies on the Rep1 protein presented here have identified distinct classes of mutations that abolish its function in plasmid maintenance. The outcomes support the current thinking that Rep1p-Rep2p as well as Rep1p-*STB* interactions are critical in plasmid partitioning. They go further to suggest that, aside from these interactions, Rep1p is likely to have additional roles in the partitioning pathway, including perhaps interactions with host encoded proteins that participate in chromosome segregation.

We have identified a set of host factors that interact directly or indirectly with the plasmid partitioning system. Several of these have turned out to be proteins that have been revealed in independent genetic screens for mutations that missegregate minichromosomes in yeast. A significant amount of our efforts was directed towards characterizing the recruitment of the yeast cohesin complex to the *STB* locus and the interaction of this complex with the Rep proteins. The association between the Rep proteins and *STB* during the cell cycle appears to be precisely timed so as to synchronize cohesin-plasmid association with cohesin-chromosome association. Based on several pieces of strong circumstantial evidence, we propose that cohesin bridges replicated sister plasmid clusters until the dissolution of this bridge during anaphase that sends the clusters towards opposite cell poles. Consistent with a role for the cellular mitotic apparatus in plasmid segregation, we have found that the integrity and compactness of the

plasmid cluster and the recruitment of the cohesin complex to *STB* are strongly compromised in the absence of a functional mitotic spindle.

Finally, by substituting the *STB* locus with LexA operator repeats and providing the Rep proteins as hybrids fused to LexA repressor, we have reconstituted the plasmid partitioning system with reasonable efficiency. Rather unexpectedly, yet strikingly, we discovered that LexA-Rep2p, in the absence of Rep1p or LexA-Rep1p, is sufficient to confer stability on the operator containing plasmid. We interpret these results to mean that the partitioning system follows the 'recruitment model', in which the central event is the functional association between Rep2p and the plasmid. When *STB* is the loading site, the Rep1 protein is an essential accessory factor for recruitment but may be dispensed (and replaced by the repressor component of the hybrid Rep2p protein) when the operator is the loading site.

What needs to be done

Future work will be directed to more critically verifying the hypothesis that has emerged from this study, namely, the plasmid steals the components of the chromosome segregation machinery for its own stable propagation. We have considered two possible models for the cohesin mediated segregation of the 2 micron circle. In one the plasmid cluster is tethered to a chromosome, and following replication, a pair of clusters bridged by cohesin stays attached to the

pair of similarly bridged sister chromatids. The cleavage of the cohesin bridge in G2/M sends one sister each carrying the attached plasmid cluster to each of the two daughter cells. The alternative model considers a chromosome independent bipolar spindle attachment and spindle mediated segregation of the plasmid clusters. Several variations of these models can be envisaged. However the critical question is whether cohesin association and dissociation can serve the equal segregation function for the plasmid. We have established that an intact mitotic spindle is essential for cohesin to be recruited to the plasmid. This finding has now been extended further through the use of mutations that differentially affect cytoplasmic versus nuclear microtubules (S. Mehta and M. Jayaram, unpublished data). Unmasking the specific role of the spindle in plasmid segregation would be an important step in establishing the intriguing connection between the spindle and plasmid-cohesin association. Finally, the recruitment model for partitioning and the implication from the present study that Rep2p (and not Rep1p) is the functional entity in plasmid maintenance need to be tested more rigorously by reconstituting the stability system using alternative strategies.

REFERENCES

Actis, L. A., Tolmasky, M. E., and Crosa, J. H. (1999). Bacterial plasmids: replication of extrachromosomal genetic elements encoding resistance to antimicrobial compounds. Front Biosci *4*, D43-62.

Adams, A. E., and Pringle, J. R. (1984). Relationship of actin and tubulin distribution to bud growth in wild-type and morphogenetic-mutant *Saccharomyces cerevisiae*. J Cell Biol *98*, 934-945.

Ahn, Y. T., Wu, X. L., Biswal, S., Velmurugan, S., Volkert, F. C., and Jayaram, M. (1997). The 2 micron-plasmid-encoded Rep1 and Rep2 proteins interact with each other and colocalize to the *Saccharomyces cerevisiae* nucleus. J Bacteriol *179*, 7497-7506.

Amon, A. (1999). The spindle checkpoint. Curr Opin Genet Dev *9*, 69-75.

Ansari, A., and Gartenberg, M. R. (1997). The yeast silent information regulator Sir4p anchors and partitions plasmids. Mol Cell Biol *17*, 7061-7068.

Araki, H., Nakanishi, N., Evans, B. R., Matsuzaki, H., Jayaram, M., and Oshima, Y. (1992). Site-specific recombinase, R, encoded by yeast plasmid pSR1. J Mol Biol *225*, 25-37.

Barton, N. R., and Goldstein, L. S. (1996). Going mobile: microtubule motors and chromosome segregation. Proc Natl Acad Sci U S A *93*, 1735-1742.

Bernard, P., and Couturier, M. (1992). Cell killing by the F plasmid CcdB protein involves poisoning of DNA-topoisomerase II complexes. J Mol Biol *226*, 735-745.

Bhat, M. A., Philp, A. V., Glover, D. M., and Bellen, H. J. (1996). Chromatid segregation at anaphase requires the barren product, a novel chromosome-associated protein that interacts with Topoisomerase II. Cell *87*, 1103-1114.

Biggins, S., Severin, F. F., Bhalla, N., Sassoon, I., Hyman, A. A., and Murray, A. W. (1999). The conserved protein kinase Ipl1 regulates microtubule binding to kinetochores in budding yeast. Genes Dev *13*, 532-544.

Bischoff, J. R., and Plowman, G. D. (1999). The Aurora/Ipl1p kinase family: regulators of chromosome segregation and cytokinesis. Trends Cell Biol *9*, 454-459.

Blat, Y., and Kleckner, N. (1999). Cohesins bind to preferential sites along yeast chromosome III, with differential regulation along arms versus the centric region. Cell *98*, 249-259.

Boeke, J. D., and Devine, S. E. (1998). Yeast retrotransposons: finding a nice quiet neighborhood. Cell *93*, 1087-1089.

Bork, P., Sander, C., and Valencia, A. (1992). An ATPase domain common to prokaryotic cell cycle proteins, sugar kinases, actin, and hsp70 heat shock proteins. Proc Natl Acad Sci U S A *89*, 7290-7294.

Bouet, J. Y., and Funnell, B. E. (1999). P1 ParA interacts with the P1 partition complex at *parS* and an ATP-ADP switch controls ParA activities. EMBO J *18*, 1415-1424.

Breeden, L. L. (1997). Alpha-factor synchronization of budding yeast. Methods Enzymol *283*, 332-341.

Brewer, B. J., and Fangman, W. L. (1987). The localization of replication origins on *ARS* plasmids in *S. cerevisiae*. Cell *51*, 463-471.

Broach, J. R., Atkins, J. F., McGill, C., and Chow, L. (1979). Identification and mapping of the transcriptional and translational products of the yeast plasmid, 2 micron circle. Cell *16*, 827-839.

Broach, J. R., Guarascio, V. R., and Jayaram, M. (1982). Recombination within the yeast plasmid 2 micron circle is site-specific. Cell *29*, 227-234.

Broach, J. R., Li, Y. Y., Feldman, J., Jayaram, M., Abraham, J., Nasmyth, K. A., and Hicks, J. B. (1983). Localization and sequence analysis of yeast origins of DNA replication. Cold Spring Harb Symp Quant Biol *47*, 1165-1173.

Buchowicz, J. (1997). Nuclear extrachromosomal DNA of higher plants. Acta Biochim Pol *44*, 13-19.

Carson, D. R., and Christman, M. F. (2001). Evidence that replication fork components catalyze establishment of cohesion between sister chromatids. Proc Natl Acad Sci U S A *98*, 8270-8275.

Castano, I. B., Brzoska, P. M., Sadoff, B. U., Chen, H., and Christman, M. F. (1996a). Mitotic chromosome condensation in the rDNA requires *TRF4* and DNA topoisomerase I in *Saccharomyces cerevisiae*. Genes Dev *10*, 2564-2576.

Castano, I. B., Heath-Pagliuso, S., Sadoff, B. U., Fitzhugh, D. J., and Christman, M. F. (1996b). A novel family of *TRF* (DNA topoisomerase I-related function) genes required for proper nuclear segregation. Nucleic Acids Res *24*, 2404-2410.

Chan, C. S., and Botstein, D. (1993). Isolation and characterization of chromosome-gain and increase-in-ploidy mutants in yeast. Genetics *135*, 677-691.

Cheeseman, I. M., Anderson, S., Jwa, M., Green, E. M., Kang, J., Yates, J. R., Chan, C. S., Drubin, D. G., and Barnes, G. (2002). Phospho-regulation of kinetochore-microtubule attachments by the aurora kinase Ipl1p. Cell *111*, 163-172.

Chen, X. J., Saliola, M., Falcone, C., Bianchi, M. M., and Fukuhara, H. (1986). Sequence organization of the circular plasmid pKD1 from the yeast *Kluyveromyces drosophilarum*. Nucleic Acids Res *14*, 4471-4481.

Ciosk, R., Shirayama, M., Shevchenko, A., Tanaka, T., Toth, A., and Nasmyth, K. (2000). Cohesin's binding to chromosomes depends on a separate complex consisting of Scc2 and Scc4 proteins. Mol Cell *5*, 243-254.

Ciosk, R., Zachariae, W., Michaelis, C., Shevchenko, A., Mann, M., and Nasmyth, K. (1998). An *ESP1/PDS1* complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast. Cell *93*, 1067-1076.

Cohen-Fix, O., Peters, J. M., Kirschner, M. W., and Koshland, D. (1996). Anaphase initiation in *Saccharomyces cerevisiae* is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. Genes Dev *10*, 3081-3093.

Dam, M., and Gerdes, K. (1994). Partitioning of plasmid R1. Ten direct repeats flanking the parA promoter constitute a centromere-like partition site *parC*, that expresses incompatibility. J Mol Biol *236*, 1289-1298.

Davis, M. A., and Austin, S. J. (1988). Recognition of the P1 plasmid centromere analog involves binding of the ParB protein and is modified by a specific host factor. EMBO J *7*, 1881-1888.

Davis, M. A., Martin, K. A., and Austin, S. J. (1992). Biochemical activities of the parA partition protein of the P1 plasmid. Mol Microbiol *6*, 1141-1147.

DiNardo, S., Voelkel, K., and Sternglanz, R. (1984). DNA topoisomerase II mutant of *Saccharomyces cerevisiae*: topoisomerase II is required for segregation of daughter molecules at the termination of DNA replication. Proc Natl Acad Sci U S A *81*, 2616-2620.

Doheny, K. F., Sorger, P. K., Hyman, A. A., Tugendreich, S., Spencer, F., and Hieter, P. (1993). Identification of essential components of the *S. cerevisiae* kinetochore. Cell *73*, 761-774.

Erdmann, N., Petroff, T., and Funnell, B. E. (1999). Intracellular localization of P1 ParB protein depends on ParA and *parS*. Proc Natl Acad Sci U S A *96*, 14905-14910.

Fang, G., Yu, H., and Kirschner, M. W. (1999). Control of mitotic transitions by the anaphase-promoting complex. Philos Trans R Soc Lond B Biol Sci *354*, 1583-1590.

Finley, J., R. L., and Brent, R. (1996). Interaction trap cloning with yeast. In D Glover and B D Hames (ed), DNA cloning-expression systems: a practical approach Oxford University Press, Oxford, United Kingdom, 169-203.

Francisco, L., and Chan, C. S. (1994). Regulation of yeast chromosome segregation by Ipl1 protein kinase and type 1 protein phosphatase. Cell Mol Biol Res *40*, 207-213.

Futcher, A. B. (1986). Copy number amplification of the 2 micron plasmid of *Saccharomyces cerevisiae*. J Theor Biol *119*, 197-204.

Gerdes, K., Gultyaev, A. P., Franch, T., Pedersen, K., and Mikkelsen, N. D. (1997). Antisense RNA-regulated programmed cell death. Annu Rev Genet *31*, 1-31.

Gerdes, K., and Molin, S. (1986). Partitioning of plasmid R1. Structural and functional analysis of the parA locus. J Mol Biol *190*, 269-279.

Gerdes, K., Moller-Jensen, J., and Bugge Jensen, R. (2000). Plasmid and chromosome partitioning: surprises from phylogeny. Mol Microbiol *37*, 455-466.

Gietz, D., St Jean, A., Woods, R. A., and Schiestl, R. H. (1992). Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res *20*, 1425.

Glaser, P., Sharpe, M. E., Raether, B., Perego, M., Ohlsen, K., and Errington, J. (1997). Dynamic, mitotic-like behavior of a bacterial protein required for accurate chromosome partitioning. Genes Dev *11*, 1160-1168.

Goh, P. Y., and Kilmartin, J. V. (1993). *NDC10*: a gene involved in chromosome segregation in *Saccharomyces cerevisiae*. J Cell Biol *121*, 503-512.

Gordon, G. S., Sitnikov, D., Webb, C. D., Teleman, A., Straight, A., Losick, R., Murray, A. W., and Wright, A. (1997). Chromosome and low copy plasmid segregation in *E. coli*: visual evidence for distinct mechanisms. Cell *90*, 1113-1121.

Hadfield, C., Mount, R. C., and Cashmore, A. M. (1995). Protein binding interactions at the *STB* locus of the yeast 2 micron plasmid. Nucleic Acids Res *23*, 995-1002.

Haering, C. H., Lowe, J., Hochwagen, A., and Nasmyth, K. (2002). Molecular architecture of SMC proteins and the yeast cohesin complex. Mol Cell *9*, 773-788.

Hartley, J. L., and Donelson, J. E. (1980). Nucleotide sequence of the yeast plasmid. Nature *286*, 860-865.

Hill, A., and Bloom, K. (1987). Genetic manipulation of centromere function. Mol Cell Biol *7*, 2397-2405.

Hiraga, S. (1992). Chromosome and plasmid partition in *Escherichia coli*. Annu Rev Biochem *61*, 283-306.

Hirano, M., Mori, H., Onogi, T., Yamazoe, M., Niki, H., Ogura, T., and Hiraga, S. (1998). Autoregulation of the partition genes of the mini-F plasmid and the intracellular localization of their products in *Escherichia coli.* Mol Gen Genet *257*, 392-403.

Hirano, T. (2000). Chromosome cohesion, condensation, and separation. Annu Rev Biochem *69*, 115-144.

Hirano, T. (2002). The ABCs of SMC proteins: two-armed ATPases for chromosome condensation, cohesion, and repair. Genes Dev *16*, 399-414.

Hirano, T., Kobayashi, R., and Hirano, M. (1997). Condensins, chromosome condensation protein complexes containing XCAP-C, XCAP-E and a Xenopus homolog of the *Drosophila* Barren protein. Cell *89*, 511-521.

Holm, C., Goto, T., Wang, J. C., and Botstein, D. (1985). DNA topoisomerase II is required at the time of mitosis in yeast. Cell *41*, 553-563.

Huberman, J. A., Spotila, L. D., Nawotka, K. A., el-Assouli, S. M., and Davis, L. R. (1987). The *in vivo* replication origin of the yeast 2 micron plasmid. Cell *51*, 473-481.

Ilves, I., Kivi, S., and Ustav, M. (1999). Long-term episomal maintenance of bovine papillomavirus type 1 plasmids is determined by attachment to host chromosomes, which is mediated by the viral E2 protein and its binding sites. J Virol *73*, 4404-4412.

Ireton, K., Gunther, N. W. t., and Grossman, A. D. (1994). spo0J is required for normal chromosome segregation as well as the initiation of sporulation in *Bacillus subtilis*. J Bacteriol *176*, 5320-5329.

James, P., Halladay, J., and Craig, E. A. (1996). Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics *144*, 1425-1436.

Janke, C., Ortiz, J., Lechner, J., Shevchenko, A., Magiera, M. M., Schramm, C., and Schiebel, E. (2001). The budding yeast proteins Spc24p and Spc25p interact with Ndc80p and Nuf2p at the kinetochore and are important for kinetochore clustering and checkpoint control. EMBO J *20*, 777-791.

Jayaram, M., Grainge, I., and Tribble, G. (2002). Site-specific recombination by the Flp protein of *Saccharomyces cerevisiae*. In Mobile DNA II, N Craig, A Lambowitz, M Gellert, and R Craigie, eds, p192.

Jayaram, M., Li, Y. Y., and Broach, J. R. (1983). The yeast plasmid 2 micron circle encodes components required for its high copy propagation. Cell *34*, 95-104.

Jayaram, M., Sutton, A., and Broach, J. R. (1985). Properties of *REP3*: a *cis*-acting locus required for stable propagation of the *Saccharomyces cerevisiae* 2 micron plasmid. Mol Cell Biol *5*, 2466-2475.

Jearnpipatkul, A., Araki, H., and Oshima, Y. (1987). A *cis*-acting locus for the stable propagation of yeast plasmid pSR1. Mol Gen Genet *207*, 355.

Jensen, R. B., and Gerdes, K. (1995). Programmed cell death in bacteria: proteic plasmid stabilization systems. Mol Microbiol *17*, 205-210.

Jensen, R. B., and Gerdes, K. (1997). Partitioning of plasmid R1. The ParM protein exhibits ATPase activity and interacts with the centromere-like ParR-*parC* complex. J Mol Biol *269*, 505-513.

Jensen, R. B., and Gerdes, K. (1999). Mechanism of DNA segregation in prokaryotes: ParM partitioning protein of plasmid R1 co-localizes with its replicon during the cell cycle. EMBO J *18*, 4076-4084.

Jensen, R. B., Lurz, R., and Gerdes, K. (1998). Mechanism of DNA segregation in prokaryotes: replicon pairing by *parC* of plasmid R1. Proc Natl Acad Sci U S A *95*, 8550-8555.

Jiang, W., and Carbon, J. (1993). Molecular analysis of the budding yeast centromere/kinetochore. Cold Spring Harb Symp Quant Biol *58*, 669-676.

Jiang, W., Lechner, J., and Carbon, J. (1993). Isolation and characterization of a gene (*CBF2*) specifying a protein component of the budding yeast kinetochore. J Cell Biol *121*, 513-519.

Kang, J., Cheeseman, I. M., Kallstrom, G., Velmurugan, S., Barnes, G., and Chan, C. S. (2001). Functional cooperation of Dam1, Ipl1, and the inner centromere protein (INCENP)-related protein Sli15 during chromosome segregation. J Cell Biol *155*, 763-774.

Kapoor, P., Shire, K., and Frappier, L. (2001). Reconstitution of Epstein-Barr virus-based plasmid partitioning in budding yeast. EMBO J *20*, 222-230.

Kikuchi, Y. (1983). Yeast plasmid requires a *cis*-acting locus and two plasmid proteins for its stable maintenance. Cell *35*, 487-493.

Kim, J. H., Kang, J. S., and Chan, C. S. (1999). Sli15 associates with the Ipl1 protein kinase to promote proper chromosome segregation in *Saccharomyces cerevisiae*. J Cell Biol *145*, 1381-1394.

Kim, J. S., and Raines, R. T. (1993). Ribonuclease S-peptide as a carrier in fusion proteins. Protein Sci *2*, 348-356.

Kim, S. K., and Wang, J. C. (1998). Localization of F plasmid SopB protein to positions near the poles of *Escherichia coli* cells. Proc Natl Acad Sci U S A *95*, 1523-1527.

Kimmerly, W. J., and Rine, J. (1987). Replication and segregation of plasmids containing *cis*-acting regulatory sites of silent mating-type genes in *Saccharomyces cerevisiae* are controlled by the *SIR* genes. Mol Cell Biol *7*, 4225-4237.

Kimura, K., and Hirano, T. (1997). ATP-dependent positive supercoiling of DNA by 13S condensin: a biochemical implication for chromosome condensation. Cell *90*, 625-634.

Kimura, K., and Hirano, T. (2000). Dual roles of the 11S regulatory subcomplex in condensin functions. Proc Natl Acad Sci U S A *97*, 11972-11977.

Kimura, K., Rybenkov, V. V., Crisona, N. J., Hirano, T., and Cozzarelli, N. R. (1999). 13S condensin actively reconfigures DNA by introducing global positive writhe: implications for chromosome condensation. Cell *98*, 239-248.

Koonin, E. V. (1993). A superfamily of ATPases with diverse functions containing either classical or deviant ATP-binding motif. J Mol Biol *229*, 1165-1174.

Laloraya, S., Guacci, V., and Koshland, D. (2000). Chromosomal addresses of the cohesin component Mcd1p. J Cell Biol *151*, 1047-1056.

Lavoie, B. D., Tuffo, K. M., Oh, S., Koshland, D., and Holm, C. (2000). Mitotic chromosome condensation requires Brn1p, the yeast homologue of Barren. Mol Biol Cell *11*, 1293-1304.

Lemon, K. P., and Grossman, A. D. (1998). Localization of bacterial DNA polymerase: evidence for a factory model of replication. Science *282*, 1516-1519.

Lewis, P. J., and Errington, J. (1997). Direct evidence for active segregation of *oriC* regions of the *Bacillus subtilis* chromosome and co-localization with the SpoOJ partitioning protein. Mol Microbiol *25*, 945-954.

Li, X., and Nicklas, R. B. (1995). Mitotic forces control a cell-cycle checkpoint. Nature *373*, 630-632.

Lin, D. C., and Grossman, A. D. (1998). Identification and characterization of a bacterial chromosome partitioning site. Cell *92*, 675-685.

Lin, D. C., Levin, P. A., and Grossman, A. D. (1997). Bipolar localization of a chromosome partition protein in *Bacillus subtilis*. Proc Natl Acad Sci U S A *94*, 4721-4726.

Livingston, D. M. (1977). Inheritance of the 2 micron plasmid from *Saccharomyces*. Genetics *86*, 73-84.

Livingston, D. M., and Hahne, S. (1979). Isolation of a condensed, intracellular form of the 2 micron plasmid of *Saccharomyces cerevisiae*. Proc Natl Acad Sci U S A *76*, 3727-3731.

Longtine, M. S., Enomoto, S., Finstad, S. L., and Berman, J. (1992). Yeast telomere repeat sequence (*TRS*) improves circular plasmid segregation, and *TRS* plasmid segregation involves the *RAP1* gene product. Mol Cell Biol *12*, 1997-2009.

Longtine, M. S., Enomoto, S., Finstad, S. L., and Berman, J. (1993). Telomere-mediated plasmid segregation in *Saccharomyces cerevisiae* involves gene products required for transcriptional repression at silencers and telomeres. Genetics *133*, 171-182.

Mead, D. J., Gardner, D. C., and Oliver, S. G. (1986). The yeast 2 micron plasmid: strategies for the survival of a selfish DNA. Mol Gen Genet *205*, 417-421.

Megee, P. C., Mistrot, C., Guacci, V., and Koshland, D. (1999). The centromeric sister chromatid cohesion site directs Mcd1p binding to adjacent sequences. Mol Cell *4*, 445-450.

Mehta, S., Yang, X. M., Chan, C. S., Dobson, M. J., Jayaram, M., and Velmurugan, S. (2002). The 2 micron plasmid purloins the yeast cohesin complex: a mechanism for coupling plasmid partitioning and chromosome segregation? J Cell Biol *158*, 625-637.

Melby, T. E., Ciampaglio, C. N., Briscoe, G., and Erickson, H. P. (1998). The symmetrical structure of structural maintenance of chromosomes (SMC) and MukB proteins: long, antiparallel coiled coils, folded at a flexible hinge. J Cell Biol *142*, 1595-1604.

Michaelis, C., Ciosk, R., and Nasmyth, K. (1997). Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. Cell *91*, 35-45.

Miki, T., Park, J. A., Nagao, K., Murayama, N., and Horiuchi, T. (1992). Control of segregation of chromosomal DNA by sex factor F in *Escherichia coli*. Mutants of DNA gyrase subunit A suppress letD (ccdB) product growth inhibition. J Mol Biol *225*, 39-52.

Mohl, D. A., and Gober, J. W. (1997). Cell cycle-dependent polar localization of chromosome partitioning proteins in *Caulobacter crescentus*. Cell *88*, 675-684.

Moller-Jensen, J., Jensen, R. B., and Gerdes, K. (2000). Plasmid and chromosome segregation in prokaryotes. Trends Microbiol *8*, 313-320.

Mori, H., Mori, Y., Ichinose, C., Niki, H., Ogura, T., Kato, A., and Hiraga, S. (1989). Purification and characterization of SopA and SopB proteins essential for F plasmid partitioning. J Biol Chem *264*, 15535-15541.

Murray, A. W., and Szostak, J. W. (1983). Pedigree analysis of plasmid segregation in yeast. Cell *34*, 961-970.

Murray, J. A., and Cesareni, G. (1986). Functional analysis of the yeast plasmid partitioning locus *STB*. EMBO J *5*, 3391-3400.

Murray, J. A., Cesareni, G., and Argos, P. (1988). Unexpected divergence and molecular coevolution in yeast plasmids. J Mol Biol *200*, 601-607.

Murray, J. A., Scarpa, M., Rossi, N., and Cesareni, G. (1987). Antagonistic controls regulate copy number of the yeast 2 micron plasmid. EMBO J *6*, 4205-4212.

Nairz, K., and Klein, F. (1997). *mre11S*--a yeast mutation that blocks double-strand-break processing and permits nonhomologous synapsis in meiosis. Genes Dev *11*, 2272-2290.

Nasmyth, K. (1999). Separating sister chromatids. Trends Biochem Sci *24*, 98-104.

Nelson, R. G., and Fangman, W. L. (1979). Nucleosome organization of the yeast 2 micron plasmid: a eukaryotic minichromosome. Proc Natl Acad Sci U S A *76*, 6515-6519.

Nicklas, R. B. (1997). How cells get the right chromosomes. Science *275*, 632-637.

Niki, H., and Hiraga, S. (1997). Subcellular distribution of actively partitioning F plasmid during the cell division cycle in *E. coli*. Cell *90*, 951-957.

Niki, H., and Hiraga, S. (1998). Polar localization of the replication origin and terminus in *Escherichia coli* nucleoids during chromosome partitioning. Genes Dev *12*, 1036-1045.

Niki, H., and Hiraga, S. (1999). Subcellular localization of plasmids containing the *oriC* region of the *Escherichia coli* chromosome, with or without the *sopABC* partitioning system. Mol Microbiol *34*, 498-503.

Niki, H., Yamaichi, Y., and Hiraga, S. (2000). Dynamic organization of chromosomal DNA in *Escherichia coli.* Genes Dev *14*, 212-223.

Nordstrom, K., and Austin, S. J. (1989). Mechanisms that contribute to the stable segregation of plasmids. Annu Rev Genet *23*, 37-69.

Ouspenski, II, Cabello, O. A., and Brinkley, B. R. (2000). Chromosome condensation factor Brn1p is required for chromatid separation in mitosis. Mol Biol Cell *11*, 1305-1313.

Ouspenski, II, Elledge, S. J., and Brinkley, B. R. (1999). New yeast genes important for chromosome integrity and segregation identified by dosage effects on genome stability. Nucleic Acids Res *27*, 3001-3008.

Ptashne, M., and Gann, A. (1997). Transcriptional activation by recruitment. Nature *386*, 569-577.

Ringrose, L., Lounnas, V., Ehrlich, L., Buchholz, F., Wade, R., and Stewart, A. F. (1998). Comparative kinetic analysis of Flp and Cre recombinases: mathematical models for DNA binding and recombination. J Mol Biol *284*, 363-384.

Robinett, C. C., Straight, A., Li, G., Willhelm, C., Sudlow, G., Murray, A., and Belmont, A. S. (1996). *In vivo* localization of DNA sequences and visualization of large-scale chromatin organization using lac operator/repressor recognition. J Cell Biol *135*, 1685-1700.

Rose, M. D., Winston, F., and Hieter, P. (1990). Methods in yeast genetics. In Cold Spring Harbor laboratory Press, Cold Spring Harbor, NY.

Ruiz-Echevarria, M. J., Gimenez-Gallego, G., Sabariegos-Jareno, R., and Diaz-Orejas, R. (1995). Kid, a small protein of the *parD* stability system of plasmid R1, is an inhibitor of DNA replication acting at the initiation of DNA synthesis. J Mol Biol *247*, 568-577.

Russell, I. D., Grancell, A. S., and Sorger, P. K. (1999). The unstable F-box protein p58-Ctf13 forms the structural core of the CBF3 kinetochore complex. J Cell Biol *145*, 933-950.

Sadoff, B. U., Heath-Pagliuso, S., Castano, I. B., Zhu, Y., Kieff, F. S., and Christman, M. F. (1995). Isolation of mutants of *Saccharomyces cerevisiae* requiring DNA topoisomerase I. Genetics *141*, 465-479.

Saitoh, S., Takahashi, K., and Yanagida, M. (1997). Mis6, a fission yeast inner centromere protein, acts during G1/S and forms specialized chromatin required for equal segregation. Cell *90*, 131-143.

Saka, Y., Sutani, T., Yamashita, Y., Saitoh, S., Takeuchi, M., Nakaseko, Y., and Yanagida, M. (1994). Fission yeast cut3 and cut14, members of a ubiquitous protein family, are required for chromosome condensation and segregation in mitosis. EMBO J *13*, 4938-4952.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sandmeyer, S. B. (1992). Yeast retrotransposons. Curr Opin Genet Dev *2*, 705-711.

Scott-Drew, S., and Murray, J. A. (1998). Localisation and interaction of the protein components of the yeast 2 micron plasmid partitioning system suggest a mechanism for plasmid inheritance. J Cell Sci *111 (Pt 13)*, 1779-1789.

Sengupta, A., Blomqvist, K., Pickett, A. J., Zhang, Y., Chew, J. S., and Dobson, M. J. (2001). Functional domains of yeast plasmid-encoded Rep proteins. J Bacteriol *183*, 2306-2315.

Sharpe, M. E., and Errington, J. (1998). A fixed distance for separation of newly replicated copies of *oriC* in *Bacillus subtilis*: implications for co-ordination of chromosome segregation and cell division. Mol Microbiol *28*, 981-990.

Shimoda, S. L., and Solomon, F. (2002). Integrating functions at the kinetochore. Cell *109*, 9-12.

Skibbens, R. V., Corson, L. B., Koshland, D., and Hieter, P. (1999). Ctf7p is essential for sister chromatid cohesion and links mitotic chromosome structure to the DNA replication machinery. Genes Dev *13*, 307-319.

Snyder, M., Sapolsky, R. J., and Davis, R. W. (1988). Transcription interferes with elements important for chromosome maintenance in *Saccharomyces cerevisiae*. Mol Cell Biol *8*, 2184-2194.

Som, T., Armstrong, K. A., Volkert, F. C., and Broach, J. R. (1988). Autoregulation of 2 micron gene expression provides a model for maintenance of stable plasmid copy levels. Cell *52*, 27-37.

Steffensen, S., Coelho, P. A., Cobbe, N., Vass, S., Costa, M., Hassan, B., Prokopenko, S. N., Bellen, H., Heck, M. M., and Sunkel, C. E. (2001). A role for Drosophila *SMC4* in the resolution of sister chromatids in mitosis. Curr Biol *11*, 295-307.

Strunnikov, A. V., Kingsbury, J., and Koshland, D. (1995). *CEP3* encodes a centromere protein of *Saccharomyces cerevisiae*. J Cell Biol *128*, 749-760.

Sudarsanam, P., and Winston, F. (2000). The *Swi/Snf* family nucleosome-remodeling complexes and transcriptional control. Trends Genet *16*, 345-351.

Sutani, T., Yuasa, T., Tomonaga, T., Dohmae, N., Takio, K., and Yanagida, M. (1999). Fission yeast condensin complex: essential roles of non-SMC subunits for condensation and Cdc2 phosphorylation of Cut3/SMC4. Genes Dev *13*, 2271-2283.

Sutton, A., and Broach, J. R. (1985). Signals for transcription initiation and termination in the *Saccharomyces cerevisiae* 2 micron plasmid. Mol Cell Biol *5*, 2770-2780.

Taketo, M., Jazwinski, S. M., and Edelman, G. M. (1980). Association of the 2 micron plasmid with yeast folded chromosomes. Proc Natl Acad Sci U S A *77*, 3144-3148.

Tam, J. E., and Kline, B. C. (1989). The F plasmid ccd autorepressor is a complex of CcdA and CcdB proteins. Mol Gen Genet *219*, 26-32.

Tanaka, T., Cosma, M. P., Wirth, K., and Nasmyth, K. (1999). Identification of cohesin association sites at centromeres and along chromosome arms. Cell *98*, 847-858.

Tanaka, T. U., Rachidi, N., Janke, C., Pereira, G., Galova, M., Schiebel, E., Stark, M. J., and Nasmyth, K. (2002). Evidence that the Ipl1-Sli15 (Aurora kinase-INCENP) complex promotes chromosome bi-orientation by altering kinetochore-spindle pole connections. Cell *108*, 317-329.

Toh-e, A., Araki, H., Utatsu, I., and Oshima, Y. (1984). Plasmids resembling 2-micron plasmid in the osmotolerant yeasts *Saccharomyces bailii* and *Saccharomyces bisporus*. J Gen Microbiol *130 (Pt 10)*, 2527-2534.

Toh-e, A., Tada, S., and Oshima, Y. (1982). 2-micron-like plasmids in the osmophilic haploid yeast *Saccharomyces rouxii*. J Bacteriol *151*, 1380-1390.

Toth, A., Ciosk, R., Uhlmann, F., Galova, M., Schleiffer, A., and Nasmyth, K. (1999). Yeast cohesin complex requires a conserved protein, Eco1p(Ctf7), to establish cohesion between sister chromatids during DNA replication. Genes Dev *13*, 320-333.

Tsuchimoto, S., and Ohtsubo, E. (1989). Effect of the *pem* system on stable maintenance of plasmid R100 in various *Escherichia coli* hosts. Mol Gen Genet *215*, 463-468.

Uhlmann, F. (2001). Chromosome condensation: packaging the genome. Curr Biol *11*, R384-387.

Uhlmann, F., Lottspeich, F., and Nasmyth, K. (1999). Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1. Nature *400*, 37-42.

Velmurugan, S., Ahn, Y. T., Yang, X. M., Wu, X. L., and Jayaram, M. (1998). The 2 micron plasmid stability system: analyses of the interactions among plasmid- and host-encoded components. Mol Cell Biol *18*, 7466-7477.

Velmurugan, S., Yang, X. M., Chan, C. S., Dobson, M., and Jayaram, M. (2000). Partitioning of the 2 micron plasmid of *Saccharomyces cerevisiae*. Functional coordination with chromosome segregation and plasmid-encoded rep protein distribution. J Cell Biol *149*, 553-566.

Voitenleitner, C., and Botchan, M. (2002). E1 protein of bovine papillomavirus type 1 interferes with E2 protein-mediated tethering of the viral DNA to mitotic chromosomes. J Virol *76*, 3440-3451.

Volkert, F. C., Wilson, D. W., and Broach, J. R. (1989). Deoxyribonucleic acid plasmids in yeasts. Microbiol Rev *53*, 299-317.

Waga, S., and Stillman, B. (1998). The DNA replication fork in eukaryotic cells. Annu Rev Biochem *67*, 721-751.

Wang, Z., Castano, I. B., De Las Penas, A., Adams, C., and Christman, M. F. (2000). Pol kappa: A DNA polymerase required for sister chromatid cohesion. Science *289*, 774-779.

Watanabe, E., Inamoto, S., Lee, M. H., Kim, S. U., Ogua, T., Mori, H., Hiraga, S., Yamasaki, M., and Nagai, K. (1989). Purification and characterization of the *sopB* gene product which is responsible for stable maintenance of mini-F plasmid. Mol Gen Genet *218*, 431-436.

Webb, C. D., Graumann, P. L., Kahana, J. A., Teleman, A. A., Silver, P. A., and Losick, R. (1998). Use of time-lapse microscopy to visualize rapid movement of the replication origin region of the chromosome during the cell cycle in *Bacillus subtilis*. Mol Microbiol *28*, 883-892.

Webb, C. D., Teleman, A., Gordon, S., Straight, A., Belmont, A., Lin, D. C., Grossman, A. D., Wright, A., and Losick, R. (1997). Bipolar localization of the replication origin regions of chromosomes in vegetative and sporulating cells of *B. subtilis*. Cell *88*, 667-674.

Weitao, T., Dasgupta, S., and Nordstrom, K. (2000). Role of the *mukB* gene in chromosome and plasmid partition in *Escherichia coli.* Mol Microbiol *38*, 392-400.

Wickner, R. B. (1992). Double-stranded and single-stranded RNA viruses of *Saccharomyces cerevisiae*. Annu Rev Microbiol *46*, 347-375.

Wigge, P. A., Jensen, O. N., Holmes, S., Soues, S., Mann, M., and Kilmartin, J. V. (1998). Analysis of the *Saccharomyces* spindle pole by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. J Cell Biol *141*, 967-977.

Wigge, P. A., and Kilmartin, J. V. (2001). The Ndc80p complex from *Saccharomyces cerevisiae* contains conserved centromere components and has a function in chromosome segregation. J Cell Biol *152*, 349-360.

Wilson, R. J., and Williamson, D. H. (1997). Extrachromosomal DNA in the *Apicomplexa.* Microbiol Mol Biol Rev *61*, 1-16.

Wong, M. C., Scott-Drew, S. R., Hayes, M. J., Howard, P. J., and Murray, J. A. (2002). *RSC2*, encoding a component of the RSC nucleosome remodeling complex, is essential for 2 micron plasmid maintenance in *Saccharomyces cerevisiae*. Mol Cell Biol *22*, 4218-4229.

Wu, H., Ceccarelli, D. F., and Frappier, L. (2000). The DNA segregation mechanism of Epstein-Barr virus nuclear antigen 1. EMBO Rep *1*, 140-144.

Wu, L. C., Fisher, P. A., and Broach, J. R. (1987). A yeast plasmid partitioning protein is a karyoskeletal component. J Biol Chem *262*, 883-891.

Zakian, V. A., Brewer, B. J., and Fangman, W. L. (1979). Replication of each copy of the yeast 2 micron plasmid occurs during the S phase. Cell *17*, 923-934.

Zakian, V. A., and Scott, J. F. (1982). Construction, replication, and chromatin structure of *TRP1 RI* circle, a multiple-copy synthetic plasmid derived from *Saccharomyces cerevisiae* chromosomal DNA. Mol Cell Biol *2*, 221-232.

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